STUDIES ON THE CEREAL GUMS

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"The living cell is above all things a heretic."

F.G.HOPKINS

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CONTENTS

GENERAL INTRODUCTION

The recognition by C. O'Sullivan (59) in 1882 of the presence of certain water-soluble polysaccharides in the grains of barley, wheat, and rye, formed a basis for future work on the cereal gums. Little further attention was directed towards this field of research until comparatively recent times. O'Sullivan's investigations led to the isolation of two polysaccharide 'bodies' termed by him the α- and β-amylans, the former being soluble in water at 40°C., the latter possessing cold water solubility. These carbohydrates were considered to consist solely of glucosan material, a relationship to starch being assumed. The methods employed by O'Sullivan for the characterization of his cereal gums did not, however, exclude the possibility of pentosan participation in their structures. Meantime the presence of pentosan in both of the amylans has been amply demonstrated but it is noteworthy that Piratzky & Wiecha (65), in 1938, described an α-amylan composed solely of glucosan material. While the nomenclature proposed by

O'Sullivan for these cereal gums is somewhat misleading, denoting as it does a close relationship to starch, the results obtained by him have in several cases received strong confirmation. Thus the isolation of some 2% of α-amylan and 0.3% of β-amylan is in agreement with the relative amounts found in later work, as are O'Sullivan's figures for wheat and rye, namely 2 - 2.5% of β-amylan and 0.05 - 0.1% of α-amylan. It should be understood that the agreement is merely qualitative due to omission of the inactivation of grain enzymes before aqueous extraction. Much smaller amounts of gums are recoverable where enzyme inactivation has been practised. The complete absence of the highly viscous α-amylan in malted barley was a further observation made by O'Sullivan, an observation, it may be added, which has successfully stood the test of time.

Lindet (41), who in 1903 reported the isolation of a barley gum possessing a highly negative optical rotation, and apparently corresponding to O'Sullivan's β-amylan, was able to show the presence of pentoses among the products

of acid hydrolysis. In 1907 Brown et al. (14), using extraction methods which were not entirely unobjectionable, lent support to Lindet's observations, thus strengthening the evidence for the presence in barley grain of water-soluble pentosan material. In this way then, a new class of substances, the water-soluble cereal gums, was brought to light although no further interest was to be bestowed upon it for a period of some thirty years.

During the time the subject of the cereal gums was lying dormant, work was steadily progressing on the study of another group of carbohydrates, the plant hemicelluloses. The term hemicellulose was first proposed by Schulze in 1891 to denote a group of substances entering into the composition of the plant cell wall. Although these hemicelluloses are similar in some respects to cellulose, the chief component of the cell wall, they differ from it in being soluble in dilute alkalies, and comparatively easily hydrolysed by dilute mineral acids to give primarily pentoses, but also hexoses and uronic acids. While the function of the hemicelluloses in the living plant

remains obscure even today, it will suffice to say for the present that they represent materials of structural importance, existing as they do in the cell wall in close association with the other components, cellulose, lignin, and the pectic substances. This association, it may be pointed out, has proved to be a stumbling block in the further investigation of the hemicelluloses, rendering as it does extremely difficult the complete separation of the latter from the other components mentioned above.

One of the first hemicellulose preparations reported was due to Tollens & Stone (85) in 1888. These workers, employing brewers' grains as source material, prepared a polysaccharide which yielded the sugars, xylose and arabinose, on hydrolysis. Four years later Schulze (79) detected the same two pentoses in the hydrolysates of hemicellulose preparations from wheat and rye brans. In the same year (1892) Schulze & Tollens (80) prepared xylan hemicelluloses from wheat straw and maize stalks. The modern period of research may be said to have opened in about the year 1921 with the work of Schryver and his associates (20) who obtained alkaline extracts of

hemicelluloses from pea-pods and effected some degree of fractionation by acidification and subsequent additions of ethanol. This procedure, with certain modifications, has been, and still is being, used by the majority of workers in the field. In 1923 O'Dwyer (56) showed the presence of xylose, arabinose, mannose, and galactose residues in hemicelluloses obtained from American white oak. Three years later the same worker (57) established the presence of uronic acid residues in some beechwood hemicelluloses. Two fractions, A and B, were obtained, the former by acidification of the alkaline extract containing the crude hemicelluloses, and the latter by the addition of ethanol to the acid solution after removal of A. Fraction A was shown to yield mainly xylose and glucuronic acid on hydrolysis, while B gave rise to arabinose, galactose, and galacturonic acid.

An important advance in fractionation technique was made by Norris & Preece in 1930 (54). Employing wheat bran as source material, after removal of pectic substances with ammonium oxalate solution and treatment with alcoholic soda to

minimise lignin content of the products, they were able to achieve a wider fractionation of hemicelluloses than had perhaps been hitherto possible. Acidification of the alkaline extract of the bran residue yielded a polysaccharide fraction A, while two further fractions, B and C, were obtained from the acid solution at acetone concentrations of about 30% and 60% by volume. Each of these fractions was subjected to further fractionation from alkaline solution with Fehling's solution whence A_1 and B_1 were recovered in the form of their copper complexes. A_2, B_2 and C_2 were obtained from the respective copper mother liquors, also as the copper complexes, by acetone precipitation. Liberation of the polysaccharides by acidification, followed by an examination of each, indicated that, with the exception of B_2 which appeared to consist substantially of glucosan material, they consisted either of pentosans or uronopentosans. Further investigations along similar lines have been carried out by Preece (66, 67, 68) employing maize cobs, brewers' grains, and boxwood, as source materials.

Some criticism has been brought to bear on

the above method of fractionation, particularly by Norman (53). The primary objection concerns the alcoholic soda treatment of the plant tissue, a procedure claimed by Norman not to be without effect upon the hemicelluloses themselves, especially when carried out at high temperatures for several hours. That this is indeed the case has been demonstrated more recently by Preece et al.(71). Further criticism has been voiced on the subject of the fractionation itself, it having been pointed out that such separation is based merely on solubility relationships and that no strong case can be produced for the classification of fractions A, B, C etc. as representing separate polysaccharides possessing different chemical properties. This much cannot be denied but it must also be borne in mind that even today many polysaccharide preparations are capable of resisting all attemps at fractionation. Only the most obviously homogeneous preparations can be attacked by the newer methylation techniques with a chance of success. Whatever the shortcomings of the Norris & Preece scheme it would appear to have served the purpose for which it was intended. While

the method has been widely adopted, the manner of application has met with some criticism from Angell & Norris (3). These workers hold to the view that hemicelluloses exhibit iso-electric precipitation, maximum yields being obtained on acidification to about pH 4. If so it would appear that, if carried out under strictly controlled conditions of pH, the A precipitation would represent a much more rigid fractionation than would that between B and C, which must remain very empirical, depending as it does merely on solubility difference. Angell & Norris also described superior hemicellulose yields through employing cupric sulphate and glycerol as precipitating agent although such has not been found to be so in certain cases (71).

Prior to 1934 almost all hemicellulose investigations had been carried out on lignified tissues. Woods, straws, oat hulls, maize cobs, and many other tissues were employed but information was scanty regarding the hemicelluloses of young non-lignified tissues. In 1934 Buston (15) turned his attention to grasses and established that the hemicelluloses in cocksfoot grass were predominantly of the galacto-araban type.

Further investigations on green tissues such as leaves and pea-pods, in 1935, yielded very similar results (16). This was obviously a point of great interest since it had previously been shown that the majority of preparations from woods etc. were of the gluco-xylan type. In general, where the galacto-araban type of hemicellulose is found, the accompanying uronide part consists of galacturonic acid residues, with glucuronic acid residues predominating in the gluco-xylan type. This state of affairs is not, however, without its exceptions.

Plant hemicelluloses are frequently rendered water-soluble by means of alkaline extraction. This may indicate either that alkaline degradation of the polysaccharides themselves has taken place or that some linkage between these hemicelluloses and other cell wall components has been ruptured. The technique employed by Cross & Bevan (23) for the separation of cell wall constituents gave rise to the conception of two hemicellulose types, namely the encrusting (polyuronide) hemicelluloses, removed together with lignin by hot sulphite extraction after preliminary chlorination of the

plant material, and the cellulosans, left behind after such treatment, in association with cellulose. These cellulosans are generally assumed to play a part in the constitution of the cell wall framework in close conjunction with cellulose. They consist mainly of mannans and xylans but glucosans are also occasionally found. However, it is the exception rather than the rule to isolate pure substances of this type, small amounts of some other sugar units or uronic acids usually being detectable. It has become increasingly doubtful whether, indeed, any sharp distinction between these hemicelluloses is justifiable, but for the present purpose the terms, encrusting hemicellulose and cellulosan, will be employed as explained above.

Little specific information is available as to the nature of the association between cellulose and the cellulosans. Irvine & Hirst (39) proposed a theory of solid solution, and other workers have postulated a certain degree of combination. In the case of xylan it is probable that the very similar relationship existing between it and cellulose renders close association relatively easy. X-ray

studies by Astbury et al. (6) support such a contention. Speculation has been perhaps more rife with respect to the association of the polyuronide hemicelluloses. These substances are acidic in character and in the isolated state can be titrated. In their native condition, however, they appear to lose such characteristics, - an indication of combination? Various investigations point to the existence of a lignin - hemicellulose linkage, the lignin perhaps existing in the enolic condition (37); the possibility of methylation must also be considered. Further support for a lignin-hemicellulose combination resides in the necessity of chlorination prior to the extraction of encrusting hemicelluloses with dilute alkali (52), in the same way as it is for removal of lignin with sulphite. It is even stated that aqueous extraction after chlorination may be successful in hemicellulose removal (53). The polyuronide hemicelluloses, which in general account for some 5-20% of the whole cell wall, consist mainly of uronopentosans, although in certain cases hexosan, particularly galactan, is found.

In view of what has been said above it is

increasingly obvious that dilute alkaline extraction will only result in the removal of a rather small proportion of the hemicellulose present. That part of the polysaccharide in close association or combination with lignin or cellulose will not be extractable under such conditions. The types of pretreatment mentioned, namely alcoholic soda and chlorination, are undesirable, but until milder means are available no thorough study of the native hemicelluloses will be possible.

With the introduction of methylation procedures and the newer chromatographic techniques more recent polysaccharide investigations have been concerned with structural determination. The most popular subject for such studies has been the xylan hemicellulose material. Present evidence indicates that two types of xylan may occur, one with and one without glycosidically bound glucuronic acid. In some cases small amounts of arabinose units are also present though whether as integral parts of a xylan molecule is somewhat uncertain. Esparto xylan prepared by repeated precipitation as the copper complex has been shown by Chanda et al. (19) to consist of about

75 xylose residues linked together by 1, 4 β - linkages and possessing one branch through a 1, 3 β-link. An algal xylan has been shown to contain xylose units linked through the 1, 3 and 1, 4 positions in the ratio of 1:4 (7, 63). Bishop (9), employing autoclave treatment to remove uronic acid and arabinose units, has given reason to suppose that certain cereal straw hemicelluloses consist of about 50 xylose residues. A very similar substance from wheat straw, consisting of 40-50 xylose units with one glucuronic acid residue attached as a side chain, is described by Aspinall & Mahomed (4). Again, Adams (1) has shown a wheat leaf hemicellulose to be built up of 88.5% xylose, 6.9% arabinose, and 5.3% uronic acid. It is unfortunate that preparative methods are not sufficiently standardised to enable direct comparison of the various hemicelluloses. It is well known that relatively mild treatment will remove arabinofuranose side chains, and recent work (11, 81) indicates an undesirable effect of alkali on polysaccharides, especially in the presence of oxygen.

The introduction of the Norris & Preece scheme for hemicellulose fractionation was not

without effect on the study of the cereal gums. Thus in 1948 Preece (69) reported the isolation of barley gums B_2 (90% hexosan) and C_2 (60% hexosan) corresponding closely to O'Sullivan's α- and β-amylans. In 1950 Preece et al. (71) made a study of barley and malt polysaccharides, reducing gum yields through prior enzyme inactivation. According to these workers three types of polysaccharide material should be considered in relation to the barley grain, namely the initially water-soluble gums, similar material in an insoluble state, and typical hemicellulosic substances rendered insoluble through association with lignin etc. Glucose, arabinose, and xylose were the constituent units of these polysaccharides, with small amounts of uronic acid residues in the hemicelluloses.

In 1951 Meredith, Bass & Anderson (47) described gums from barley, malt, and wort, in yields approximately three times greater than obtained by Preece et al. (71), due to omission of enzyme inactivation. The products obtained in the two cases, while yielding similar sugar units, cannot be strictly comparable. Anderson (35) has since

noted the value of inactivation and further interesting investigations have been reported by this group of Canadian workers (36, 48, 8).

About this time Perlin (64) advanced a structure for a water-soluble arabo-xylan prepared from wheat flour. This he envisaged to consist of a straight chain of xylose units linked through the 1 and 4 positions, with single arabinose residues attached glyosidically as side chains through the 2 and 3 positions of the xylose residues. The solubility of such a molecule is apparently increased by a decrease in the ratio of xylose to arabinose units. It is likely that this pentosan represents a purer form of that formerly described by Ford & Peat (33). Structural studies on barley gum have been reported by Gilles, Meredith & Smith (34) who, by fractionation after methylation, obtained a methylated arabo-xylan of high negative rotation, a methylated poly-α-glucosan of high positive rotation, and a poly-β-glucosan of low negative rotation.

There can be no doubt that one of the major deterrents to polysaccharide study is the difficulty inherent in the resolution of mixtures into homogeneous or near-homogeneous substances. Remarkable

progress has been made in this direction by the application of the salt fractionation procedure of Preece & Mackenzie (72) to barley and malt gums. In this way a pure glucosan fraction, conveniently termed β-glucosan, was obtained from barley grain. Structural investigations (5) indicate the presence of 1, 3 and 1, 4 β-linkages. There is ample proof that O'Sullivan's α-amylan and Preece's B_2 gum are composed principally of this β-glucosan. Also, in view of O'Sullivan's failure to isolate α-amylan from malt, it is interesting that β-glucosan has been shown to be absent from this source (72). Besides this β-glucosan fraction several pentosan-rich preparations have been made from barley. It is not unlikely that arabo-xylans such as that found in wheat (64) will be present in barley, although no hexosan-free fraction has, as yet, been obtained from this source. The salt fractionation technique offers great possibilities and its mild nature would appear to render it preferable, where applicable, to the various alkaline treatments. The theory of such a precipitation is at present obscure.

A preliminary survey of unfractionated wheat,

rye, oat, and maize gums was made by Preece & Mackenzie (73). The results showed oats and maize, like barley, to be rich in glucosan material. However, the barley gums exhibited negative optical rotations whereas the others were dextrorotatory. Wheat and rye grain on the other hand were found to be relatively rich in laevo-rotatory pentosan gum of fairly high viscosity in aqueous solution. Also, investigation of fractionated barley and wheat grain strongly suggested the endosperm to be the chief site of location of these water-soluble gums.

Recent work by Meredith et al. (49) indicates the desirability of complete enzyme inactivation in barley grain with alcohol treatment followed by the action of a papain preparation. By this means a gum of extremely high viscosity has been produced consisting chiefly of glucose, arabinose, and xylose residues. A very small nitrogen content is always reported although it is uncertain whether this represents an integral part of the gum molecule.

The observation by Brown & Morris (12) in 1890, of the degradation of barley endosperm cell

walls during germination, was one of the first indications that mechanisms capable of carrying out such a transformation existed. There is a justifiable inclination to consider the plant cell wall as constituting an end product of protoplasmic activity. This appears to be true to some extent since no plant enzymes are known with certainty to be capable of acting upon the cell wall cellulose. However, before starch breakdown in the grain endosperm can occur, and before products can be transported out of the cells, some transformation of the walls will be necessary.

Preliminary investigations of cell wall metabolism, and of the enzymes concerned, have generally been made by means of analysing plant tissues at various stages of growth for individual cell wall components. Unfortunately, in several cases such studies have resulted in theorising to an extent unjustified by the results obtained. Such views for example, as those forwarded for various transformations between lignin, pectin (the pectic materials), hemicelluloses, and cellulose, while attractive to a certain extent, are woefully lacking in experimental support. In

view of present day knowledge it seems much more likely that polysaccharides of the type considered here will prove to be synthesised from small carbohydrate units by way of the known metabolic cycles. Even so, however, the possibility that starch may act as a precursor of certain hemicelluloses (18, 58) seems worthy of mention. It is uncertain whether hemicelluloses are of use to the plant other than in a structural capacity although Buston (16) has indicated that they may be partially used up in starving detached leaves. Burkhart (14) has suggested that such substances may act as a reserve food supply in alfalfa roots.

While enzymes deemed to be specific for certain pure polysaccharide substrates have been described (e.g. arabanase, xylanase). preliminary work on the cereal polysaccharides has been carried out employing preparations which are almost certainly mixtures of enzymes termed cytases. The chief reason for the lag in enzymic studies is the difficulty residing in the preparation of a homogeneous substrate. Preece et al. (71), using unfractionated gum and hemicellulose preparations as substrate for enzymes extracted from raw,

steeped, and malted barley, have presented information to support the presence of at least two enzyme systems. One of these, a cytoclastic or disaggregating system producing but a small liberation of reducing groups from its substrate, was found to be present in the raw barley grain; the other, a cytolytic system producing large numbers of reducing groups from the breakdown products of the first, appeared to accumulate on autolysis or on germination of the barley grain. Sandegren & Enebo (78), using a synthetic substrate, ethyl hydroxyethyl-cellulose, have contrived to follow the action of some cytases by means of viscosity drop. This procedure, while praiseworthy in itself, cannot be wholly satisfactory in view of the deviation from the use of a naturally occurring substrate. The same authors (32) have described two enzyme systems in green malt, one of which is active in splitting 1, 4 β -linkages of their substrate, the other apparently being concerned with transglycosidation. With the isolation of β-glucosan (72) the possibility of the understanding of some of the cytase enzymes has been greatly increased.

Preliminary investigations have been made by Preece & Aitken (74) employing this natural substrate.

While at present the structure and metabolism of the plant cell wall is only poorly understood it is true to say that interest in the topic has greatly increased in recent years. There seems no reason to doubt that a concerted effort on the part of the investigators, in the application of newer and better experimental techniques, will eventually prevail in the elucidation of the problem.

FRACTIONATION OF THE WATER-SOLUBLE CEREAL GUMS

INTRODUCTION

On various occasions information has become available on the nature of the cereal gums. Thus, Meredith *et al.* (8, 35, 36, 47, 48, 49) have made the barley gums the focus of their attention, while Perlin (64) has published interesting material on the wheat gums. The work carried out by Preece *et al.* (71, 69, 72, 74) has also been largely concerned with the gums of a single cereal, namely barley. More recently, however, Preece & Mackenzie (73) have obtained some information pertaining to the gums of other cereals through studying some unfractionated gums prepared by precipitation with Fehling's solution and acetone. In view of the interesting results obtained by the latter workers (72), from the application of a salt fractionation technique to barley and malt gums, it has become of interest to extend this fractionation procedure to the gums of rye, wheat, oats, and maize. By so doing it is believed that a more direct comparison of the various gums can be made than has hitherto been possible. It has frequently been noted that

gums prepared from different sources by different workers, employing various methods of approach, have formed the subject for comparison. Such preparations are not necessarily directly comparable, it being highly desirable to employ one technique for the purpose, thus minimising the variables inherent in any such procedure.

Besides the more academic interest aroused by the cereal gums, the industrial significance of these substances has been the subject of papers by Pence, Elder, & Mecham (62), and by Clendenning & Wright (21). The former point out the influence of pentosans on the consistency of doughs in the baking industry whereas the latter indicate the interference of highly viscous pentosans during starch manufacture from wheat.

It is hoped then to gain further information as to the nature of the cereal gums in a more general sense, and by so doing to assist in elucidating certain problems which have arisen, both in the industrial and academic fields.

EXPERIMENTAL

Materials:- The four cereals employed in this investigation represented current commercial samples. In the tables of results shown below some earlier results (72) for the barley gums are given for comparison. However, some new figures are also included for this cereal. In all cases the raw grain was used.

Preparation of extracts:- Normally 2 or 3 kg. quantities of grain were worked up. This was submitted to a fairly fine grinding in a coffee mill after which it was slowly added to, and thoroughly mixed with, twice its weight of boiling 80% aqueous ethanol. The mixture was refluxed on a water bath for 30 min. and then filtered hot through muslin. The process was repeated using the grain residue which was finally air-dried.

For the purpose of extraction the dry grain residue was stirred with 3 x 2½ times its weight of water at 40°C. for 3 successive 30 min. periods. A little crystalline thymol was always added as antiseptic during this time. After each extraction the grain residue was filtered off on muslin and the filtrates combined. The extract was then

centrifuged at 3,000 r.p.m. for periods of 10 -30 min., depending on the viscosity, in order to remove most of the starch present. For the complete removal of the residual starch the centrifugate was filtered bright through acid-washed kieselguhr on a double thickness of Whatman No. 1 filter paper. It was normally necessary to filter twice before clarity was attained. The filtration entailed considerable time and difficulty, especially in the case of the very viscous extracts obtained from rye and wheat. The filtered extract was taken down to a volume of 500 - 1000 ml., the volume depending on the initial extract viscosity, in large porcelain basins on boiling water baths. On evaporation a fairly sticky white skin was found to form on the solution surface in some cases, and this, after completed evaporation, was centrifuged off, extracted twice with boiling water, and the extract combined with the main solution. The whole was filtered bright through kieselguhr and was, at this stage, ready for fractionation. Some preparations were made from rye omitting the evaporation step. In such cases the aqueous extract, after filtration, etc.,

was treated with acetone, the gum precipitated, and redissolved in water to give a solution ready for fractionation. While rye and wheat extracts were found to exhibit extremely high viscosities those from oats and maize possessed only very low ones. The above-mentioned skin apparently represents gum dehydrated by surface evaporation.

Fractionation:- This was carried out in the manner originally described by Preece & Mackenzie (72), with two main modifications, the first of which constitutes fractionation at a constant temperature of 15°C. This procedure was found to decrease markedly the spread in precipitation observed where such precautions were not taken. Solid ammonium sulphate was slowly added to the continuously stirred extract, 20 g. salt/100 ml. extract being added in the first instance. Any precipitate thrown down was centrifuged off at 3,000 r.p.m. for a period of up to 30 min. depending on the state of the gum. In some cases, notably where rye was the gum source, and especially where prior extract evaporation had not been practised, it was found necessary to allow the salt solution to stand overnight before centrifuging,

thus enabling coagulation of the precipitate. Further additions of salt were made to give concentrations of 30, 40, 50, 60, and 70%, at which final concentration the solution was saturated with respect to ammonium sulphate. The saturated mother liquor was reserved for separate working up. Each fraction, after separate solution in water at about 80°C., and cooling of the solution, was reprecipitated with acetone, this treatment removing a great deal of the colouring matter present. After separate re-solution the gums were normally subjected to five further fractionations as described by Preece & Mackenzie (72), and as described above for the first precipitation. After the third precipitation each solution was filtered bright and precipitated with acetone. Where gum yields were very small only four fractionations were practised, it having become increasingly obvious that continued reprecipitation caused increased gum loss and decreased viscosity of the products. On completion of fractionation the separate precipitates were dissolved in water and dialysed in cellophane tubes against running tap water for three days, thymol always being added to

the tubes. On the completion of dialysis the gum solutions were filtered bright and precipitated with acetone (final concentration not less than 60%). After removal of liquid by centrifuging, each fraction was allowed to stand with three changes of 95% ethanol for three 1 hr. periods, the gums then being filtered off and dried in vacuo for 2 - 3 days over calcium chloride or phosphorus pentoxide. They were then powdered in a mortar and stored in well stoppered tubes. The mother liquor obtained from the initial salt precipitation was dialysed as for the gum fractions until no sulphate ions were detectable. It was then taken to a small volume (50 - 100 ml.) on a water bath, filtered bright, and treated with acetone (400 - 800 ml.). The precipitate, which was usually of an extremely sticky nature, was taken to dryness as above. This latter procedure constitutes the second important modification of Mackenzie's method, he evaporating the salt-saturated solution prior to dialysis. In the course of the present work this procedure was attempted with little success, due probably to evaporation at the pH of 4.6 given by saturated

ammonium sulphate solution, or to the presence of the inorganic ions themselves.

The method of fractionation adopted usually resulted in the preparation of gums virtually completely precipitable at their respective salt concentrations. In one case, however, an oat gum was recovered which precipitated between 20 and 30% salt on successive fractionations. This was reserved separately and may bear some relationship to the 30 - 40 fraction obtained from barley by Mackenzie (72).

Properties and compositions of the fractions:- The products were white, fibrous or pulverulent substances, soluble in water on heating, or on prolonged stirring at room temperature giving, normally, clear solutions. The mother liquor fractions (non-precipitable by ammonium sulphate) were white or cream coloured powders dissolving immediately in cold water to give clear solutions. Moisture contents were determined by heating for a 2 hr. period at 100°C. and ash contents by ignition in porcelain crucibles. Only in a few of the mother liquor fractions were ash contents greater than 1% encountered. Even in these cases the results

represented considerable improvement over the older method of preparation. The normal ash content was less than 0.5% and moisture contents lay in the range of 8 - 12%. Yields of the fractions are given in Table I. Specific rotations (0.5% aqueous solution at 15°C. in 1 dm. tube) and specific viscosities (0.5% aqueous solution at 25°C) are shown in Table II. The latter were determined in a thermostatically-controlled water bath at 25°C. ± 0.01° using British Standard Ostwald viscometers (time of flow for water ca. 20 sec.). The products of gum hydrolysis were investigated by descending paper partition chromatography (22) employing the general technique described by Partridge (60). After suitable treatment, to be described below, gum hydrolysates were applied to paper and dried at 30 - 40°C. After running, the papers were dried at 100°C. for 15 mins. and carbohydrates were detected by spraying with aniline oxalate (61) and developing at 100°C. for periods of 5 - 10 min. For quantitative estimination of the sugar units 10 mg. portions of the gums were hydrolysed with 10 ml. N H_2SO_4 under reflux for 3 hr. Originally, cooled acid

Table I

Yields of Higher-Molecular Cereal Gum Fractions

$(NH_4)_2SO_4$ precipitation level (%)	Yields (dry, ash-free), % of dry grain				
	Rye	Wheat	Barley	Oats	Maize
20	-	-	0.380[*]	0.061	-
20 - 30	-	-	-	0.141	-
30	0.020	0.038	0.037[*]	0.037	-
30 - 40	-	-	0.023[*]	-	-
40	0.071	0.063	0.034[*]	0.012	0.001
50	0.132	0.025	0.035[*]	-	0.004
60	0.020	0.005	-	-	-
Saturation	-	-	0.010[*]	0.009	-
Mother Liquor [†]	0.243	0.157	0.048	0.082	0.132
Total recovery	0.49	0.29	0.57	0.34	0.14

[†] Products recovered by acetone precipitation after dialysis.

[*] Results of Preece & Mackenzie (72).

Table II

Physical Properties of Gum Fractions

$(NH_4)_2SO_4$ precipitation level (%)	Specific rotation (°) (0.5% soln., 15°C)					Specific viscosity (0.5% soln., 25°C)				
	Rye	Wheat	Barley*	Oats	Maize	Rye	Wheat	Barley*	Oats	Maize
20	-	-	-11	-2	-	-	-	1.88	1.44	-
20-30	-	-	-	-8	-	-	-	-	1.15	-
30	*	-110	-10	-16	-	3.18	4.25	0.66	0.59	-
30-40	-	-	-36	-	-	-	-	1.22	-	-
40	-135	-100	-128	-20	*	2.37	2.80	3.52	0.59	*
50	-131	-68	-141	-	+112	1.89′	2.00	1.90′	-	0.16
60	-136	*	-	-	-	2.76	*	-	-	-
turation	-	-	-98	*	-	-	-	0.72	0.78	-
ther liquor	-18	-18	+18	-4	+126	0.10′	0.04′	0.10′	0.05′	0.05′

* Not determined
′ Denotes pulverulent product, all others being fibrous.
* Results of Preece & Mackenzie (72) with the exception of the mother liquor fraction.

hydrolysates were neutralised with barium hydroxide but much more complete recovery (95 - 100%) of sugar units has been obtained by neutralising with N NaOH; methyl orange, and more recently methyl red, being used as indicator, in minimal quantity. For good chromatographic separation it is necessary to remove the sodium sulphate formed, with 4 vols. of ethanol, thorough shaking, and standing for 10 min. to ensure complete precipitation prior to filtration. The alcoholic filtrate can then be taken to a small volume in vacuo (40°C), applied to the paper in bands by means of a glass chromatographic pipette, dried at 30 - 40°C., and chromatographed. The standard solvent system employed for this purpose was butanol:acetic acid:water (40:10:50). Longitudinally cut strips from the chromatograms were sprayed, the individual sugars located, and eluted by the method of MacLeod (43), prior to estimation by the copper reduction method of Somogyi (82), employing standard curves constructed for the purpose. Mixed spots, such as those formed by glucose and galactose in the above solvent system, were eluted, and resolved in phenol-water. In all of the quantitative chromatographic work described in this section, Whatman

No. 1 filter paper was employed, Watman No. 4 occasionally being used for qualitative runs. Unless otherwise stated this method of chromatographic procedure can be assumed to have been employed throughout the whole of the present work. Qualitative chromatograms of particular interest are shown in Plates I and II. The total yields of the various anhydro-sugar units are shown in Table III, the figures being summations of yields from individual fractions; in this connection see also Fig. I. The major units identified in these gums are glucosan, galactan, araban, and xylan. These terms, it should be noted, are used for the sake of convenience and do not necessarily assume the existence of single polysaccharides. No mannose was detected in the hydrolysate of any of the present fractions. Faint pink (pentose) spots (R_F values 0.30 and 0.34 respectively in butanol: acetic acid:water) have been observed on chromatograms of the rye and wheat gum 50% fractions. These may be artifacts arising from xylose due to the experimental technique or they may be due to traces of other true pentose sugars. Similar observations were made by Preece & Mackenzie (72)

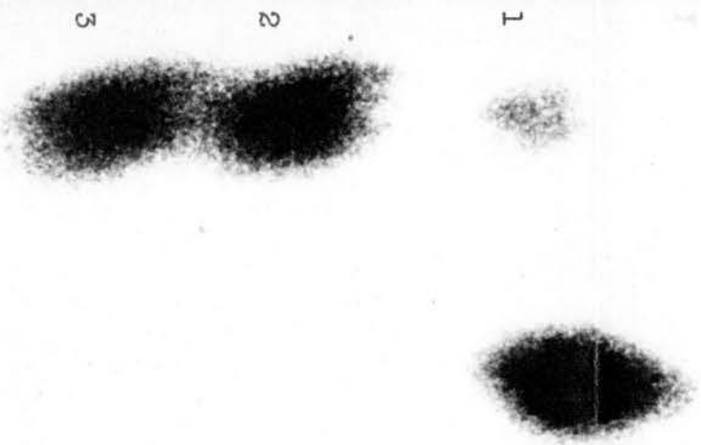
PLATE I

Plate I. Acid hydrolysis products of oat(20) and rye(40) gums.

1 glucose

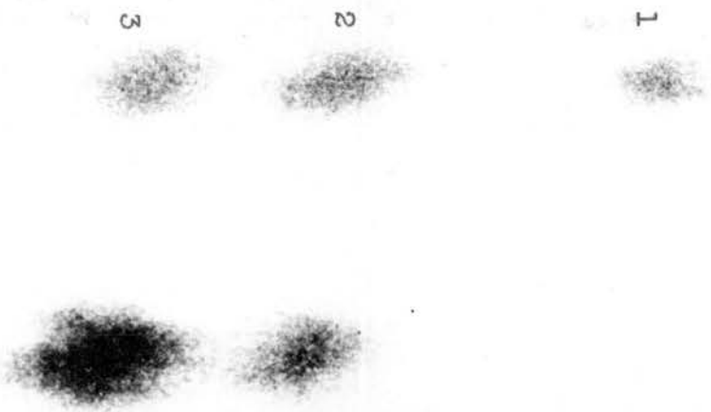
2 arabinose

3 xylose



CONTROL

WATER SOL.
OAT GUM.
(20% $(NH_4)_2SO_4$)



CONTROL

WATER SOL.
RYE GUM
(40% $(NH_4)_2SO_4$).

PLATE II

Plate II. Acid hydrolysis products of an oat husk hemicellulose fraction(70) and an oat mother liquor gum fraction.

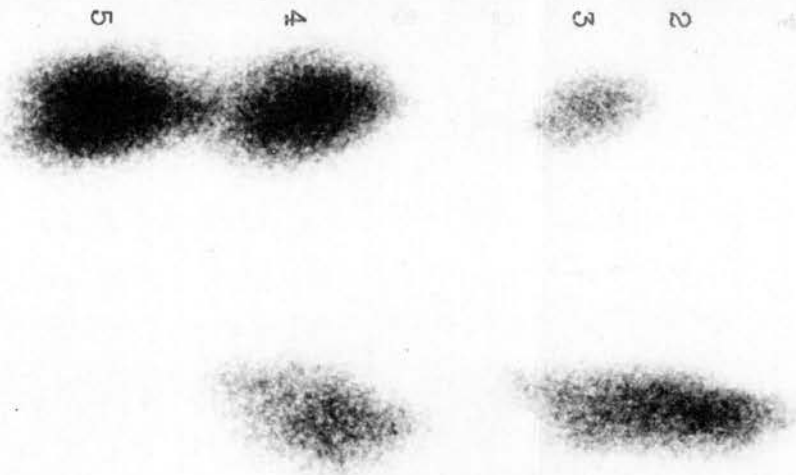
1 galacturonic acid

2 galactose

3 glucose

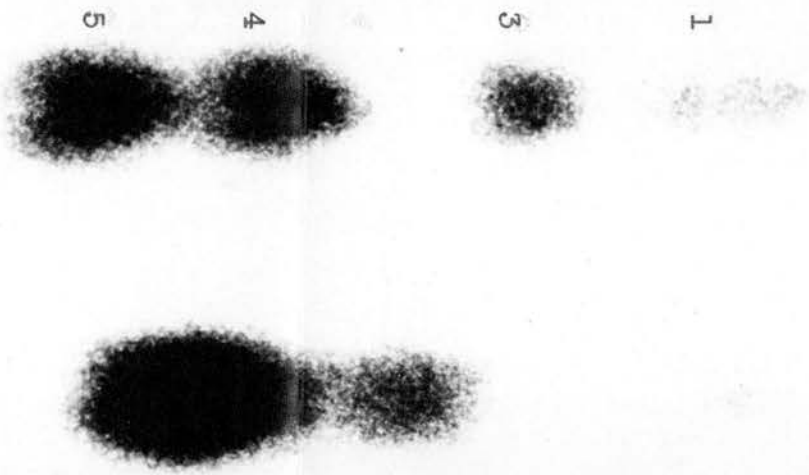
4 arabinose

5 xylose



CONTROL

WATER SOL.
OAT GUM
(ACETONE
PPTBLE.)



CONTROL

OAT HUSK
HEMICELL.
(50-70%
$(NH_4)_2SO_4$)

Table III

Yields of Anhydro-Sugar Units
(Results are totals of all fractions, % of dry grain)

Unit	Rye	Wheat	Barley	Oats	Maize
Glucosan	0.062	0.446	0.444	0.262	0.090
Xylan	0.155	0.089	0.057	0.014	0.010
Araban	0.196	0.115	0.057	0.035	0.025
Mannan	0.000	0.000	0.001	0.000	0.000
Galactan	0.058	0.042	0.006	0.025	0.010

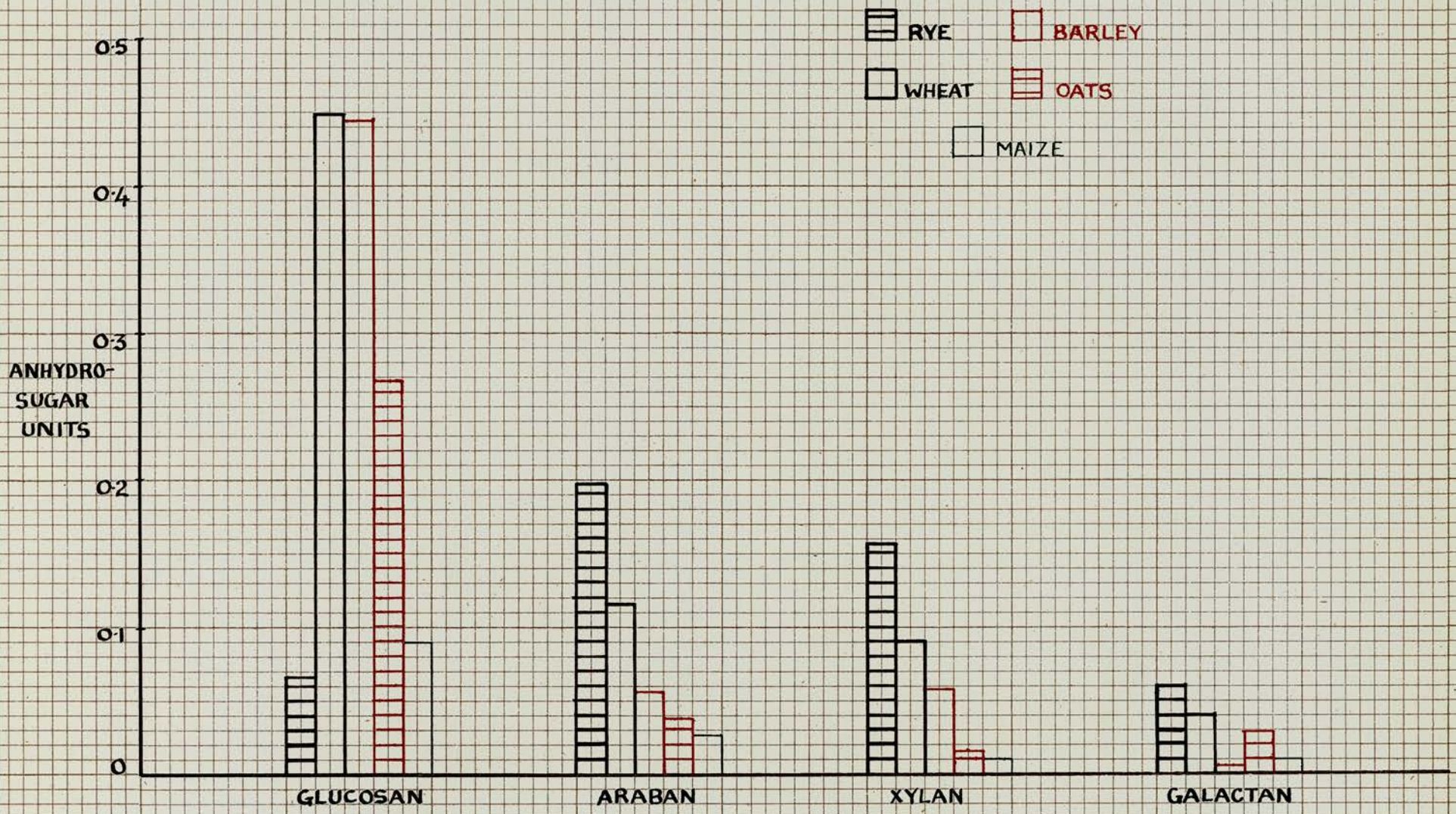


Figure I. Water-soluble cereal gum sugar units as a % of the dry grains.

for some pentosan-rich fractions. The hydrolysate of the oat gum (mother liquor fraction) contained two products of partial hydrolysis. These gave brown and pink spots (aniline oxalate) having R_F values 0.05 and 0.06 respectively, in butanol: acetic acid:water. Incidentally, it should be noted that R_F values shown were calculated relative to glucose (R_F 0.17), arabinose (R_F 0.21) or xylose (R_F 0.24), as experimentally determined.

Distribution of the units:- The approximate compositions of the fractions are shown in Table IV, a number of interesting features revealing themselves. The use of butanol:acetic acid:water rather restricts the accuracy of the results since separation between arabinose and xylose is not so clear as that obtained with other solvents such as butanol:ethanol:water. However, the former solvent affords a more rapid separation and duplicate estimations indicate errors in the region of $\pm$ 5%, tolerable where strictly quantitative results are not necessary.

The action of malt α-amylase on glucosan containing gum fractions:- The amylase preparation was made from malt by the method of Preece & Shadaksharaswamy (70). Briefly, the method consists of extraction of ground malt with 20%

Table IV

Composition of the Principal Gum Fractions
(Results shown are the approximate percentage compositions of the fractions)

$(NH_4)_2SO_4$ precipitation level (%)	Unit	Rye	Wheat	Barley*	Oats	Maize
20	Glucosan	-	-	100	100	-
20-30	Glucosan	-	-	-	100	-
30	Glucosan	17	11	96	93	-
	Araban	38	25	2	6	-
	Xylan	45	64	2	1	-
40	Glucosan	0	6	12	88	40
	Araban	39	33	32	4	19
	Xylan	61	61	56	8	40✓
50	Glucosan	0	23	7	-	92
	Araban	45	30	28	-	6
	Xylan	55	47	65	-	0+
60	Glucosan	2	56	-	-	-
	Araban	30	20	-	-	-
	Xylan	68	24	-	-	-
Saturation	Glucosan	-	-	17	61	-
	Araban	-	-	9	19	-
	Xylan	-	-	61	20	-
	Mannan	-	-	13	0	-
Mother liquor	Glucosan	25	18	28	15	66
	Araban	42	47	42	40	19
	Xylan	9	9	5	14	7
	Galactan	22	26	25	31	8

* With the exception of the mother liquor fraction the results quoted for barley are those of Preece & Mackenzie (72)
✓ ca. 1% galactan + ca. 2% galactan

ethanol, precipitation of the crude enzyme at 60% ethanol, and inactivation of β-amylase by heating in aqueous solution at 70°C. with calcium acetate. 120 mg. of the amylase preparation were obtained from 200 g. of malt. The action of this preparation was studied, employing some glucosan-rich gum fractions as substrates, more especially those fractions found to give a coloration with iodine. In each case observations were made on the gum before and after enzyme action.

For the purpose of this investigation 0.5 g. portions of gum or starch (used as control) were made up to 48 ml. in aqueous solution and buffered with 2 ml. acetate buffer pH 4.6. 5 or 10 mg. of the amylase preparation were dissolved with stirring in a mixture of 8 ml. water and 2 ml. acetate buffer pH. 4.6 at room temperature. Substrate and enzyme solutions were then brought to 21°C., mixed, and incubated at 21°C. in the presence of a little crystalline thymol. Where possible, reactions were followed by means of iodine coloration tests. Controls, run simultaneously, contained 10 ml. water-buffer mixture in place of the enzyme solution. 5 ml. portions of the

reaction mixtures were withdrawn at intervals, brought to 100°C. to stop enzyme action, concentrated to a syrup on a water bath, and partitioned qualitatively on Whatman No. 1 paper in butanol: acetic acid:water as described above. Specific rotations of several of the fractions were measured before and after amylase action, the latter after dialysis of the reaction mixture and recovery of the gum by acetone precipitation. The results are summarised in Table V. In conjunction with these determinations, an attempt was made to estimate the recovery of gum in a higher-molecular form after enzyme action, by means of acetone precipitation after dialysis. It soon became evident, however, that this problem was much deeper than was at first suspected. Thus, even straightforward precipitation of gums from aqueous solution was found to be accompanied by appreciable losses; 20 - 30% in the case of pentosan; ca. 10% in the case of β-glucosan; i.e. much more than could be accounted for by dextrinous glucosan in the fractions. Even larger losses were obvious for the very soluble mother liquor fractions. Determination of gum viscosities before and after anzyme action

Table V.

α-Amylase Action on some Glucosan-containing Gum Fractions*

Gum fractions χ	Incubation time (hr.)	Iodine coloration (before)	Iodine coloration (after)	Sugars detected	$[\alpha]_D$ before incubation (o)†	$[\alpha]_D$ after incubation (o) †
Malt(acetone)	23	orange-brown	-	maltotriose maltose,glucose	+22°	+14
Maize (acetone)	23	violet	-	" " "	+126	+86
Wheat (50)	23	red-brown	-	traces of hexoses	-68	-112
Wheat (30)	21	deep blue	slight green	≠	-110	-134
Barley (20)χ	21	-	-	-	≠	≠
Barley (30)	40	-	-	-	≠	≠
Starch	21	intense blue	-	maltrotriose maltose,glucose	≠	≠

* All blanks showed no sugars or oligosaccharides on chromatograms.

χ 5 mg. α-amylase preparation; in all other cases 10 mg.

† 0.5% solution at 15°C.

≠ not determined.

χ Figures in brackets refer to % salt used in preparation; acetone refers to mother liquor fractions.

encountered similar difficulties, it being found extremely troublesome to prepare from a pentosan-rich gum two solutions of the same concentration, possessing equal viscosities. It is noteworthy that this problem does not arise to anything approaching the same extent in the case of β-glucosan. A more detailed consideration of these problems is presented in Section V while the results in Table V are merely qualitative. While this investigation was carried out for the purpose of differentiating between α-glucosan (starchy or dextrinous) material, and β-glucosan, it is not certain whether the amylase preparation possessed pentosanase activity. Information concerning the relative amounts of sugar units in pentosan-rich fractions before and after amylase action certainly indicates that the xylan:araban ratios remain unchanged throughout. Also, recent work (2) indicates that little, if any, gum degrading enzyme would withstand the heat treatment with calcium ions (see also Section VI). The absence of pentose and hexose oligosaccharides from chromatograms of amylase action on pentosan-rich and β-glucosan fractions respectively, may agree with

the above information in characterizing losses as mechanical rather than as being due to enzymic degradation of non-glucosan material.

Partial acid hydrolysis of some gum fractions:-

By hydrolysis with acid of varying normality an attempt has been made to demonstrate some degree of correlation between various gum fractions. The general procedure consisted in hydrolysing 2 ml. portions of 0.5% aqueous solutions of the gums with 8 ml. of standard acid (H_2SO_4) in glass-stoppered pyrex glass tubes for 2 hr. periods in water baths at 50 or 100°C. After cooling the hydrolysate, and neutralizing to methyl red with sodium hydroxide of normality corresponding to that of the acid employed, 5 ml. of the solution were treated as usual and chromatographed on Whatman No. 1 filter paper against suitable control sugars. The solvents employed were butanol:acetic acid:water (40:10:50) and butanol:ethanol:water (45:5:50), the latter containing 1% by weight of ammonia in the aqueous phase. In the case of the ethanol solvent, commercial butanol was redistilled for use and ethanol was purified by distilling from NaOH, drying over and distilling from $KHCO_3$, and

drying over and distilling from $CaCl_2$. This butanol:ethanol solvent, although slower running than the other, is much more stable and results in less variation in R_F values.

The hydrolytic procedure was applied to several gum fractions, chromatographic results being presented in Table VI. Chromatograms are shown in Plates III and IV. The results indicate a greater ease in acid hydrolysis of pentosan as compared with β-glucosan. The hexose oligosaccharides of R_F values 0.09 and 0.11 in butanol: acetic acid:water, and 0.020 and 0.033 in butanol: ethanol:water, correspond to cellobiose and laminaribiose respectively, in both solvents. No great concentration of these oligosaccharides could be produced, thus barring the way to further investigation. However, interesting information has been obtained on the nature of two pentose oligosaccharides produced by hyrolysis of the wheat and rye gums. One of these, that having R_F values of 0.11 and 0.033 in butanol:acetic acid and butanol: ethanol respectively, was obtained by partition of the pentosan hydrolysates (0.1 N H_2SO_4) in butanol:acetic acid. Enough was recoverable from

Table VI

The Partial Hydrolysis of some Gum Fractions

(H denotes hexosan; P denotes pentosan material)

Gum fraction*	Normality of H_2SO_4	Butanol:acetic acid:water		Butanol:ethanol:water	
		Carbohydrates on chromatograms	R_F value#	Carbohydrates on chromatograms	R_F value#
Barley(30) Oat (20)	0.5+	H	0	H	0
	0.1	H	0	H	0
		glucose		glucose	
	0.2	H	0	H	0
		H	(diffuse)	H	0.004
		H	0.09	H	0.020
		H	0.11	H	0.033
		glucose		glucose	
	0.5	H	0	H	0
		H	0.09	H	(diffuse)
		H	0.11	H	0.033
		glucose		glucose	
Rye (40) Wheat (40)	0.1	P	0	P	0
		P	0.07	P	0.006
		P	0.12	P	0.036
		arabinose		trace P	0.095
		xylose		arabinose	
				xylose	
	0.2	P	0	P	0
		P	0.07	P	0.006
		P	0.12	P	0.036
		arabinose≠		trace P	
		xylose		arabinose≠	
				xylose	
Rye (40)	0.02			P	0
				P	0.005
				P	0.035
				arabinose	
				xylose	

* See Table V.
\- Normality of acid used for hydrolysis of 2 ml. gum soln.
\+ Temperature of hydrolysis, 50°C.; all others 100°C.
≠ Glucose visible in wheat (40)
\# Values calculated from glucose R_F -0.105, arabinose R_F - 0.163
xylose R_F - 0.205

PLATE III

Plate III. Partial acid hydrolysis of cereal pentosan gums.

(Solvent, butanol:ethanol:water, 1% NH_3 by wt. in aq. phase)

Rye(40)

A 0·1N H_2SO_4 2hr. 100°C

B 0·2N H_2SO_4 2hr. 100°C

1 galacturonic acid

2 cellobiose

3 laminaribiose

4 maltose

5 glucose

6 arabinose

7 xylose

8 pentose oligosaccharides

9 xylotriose

10 xylobiose

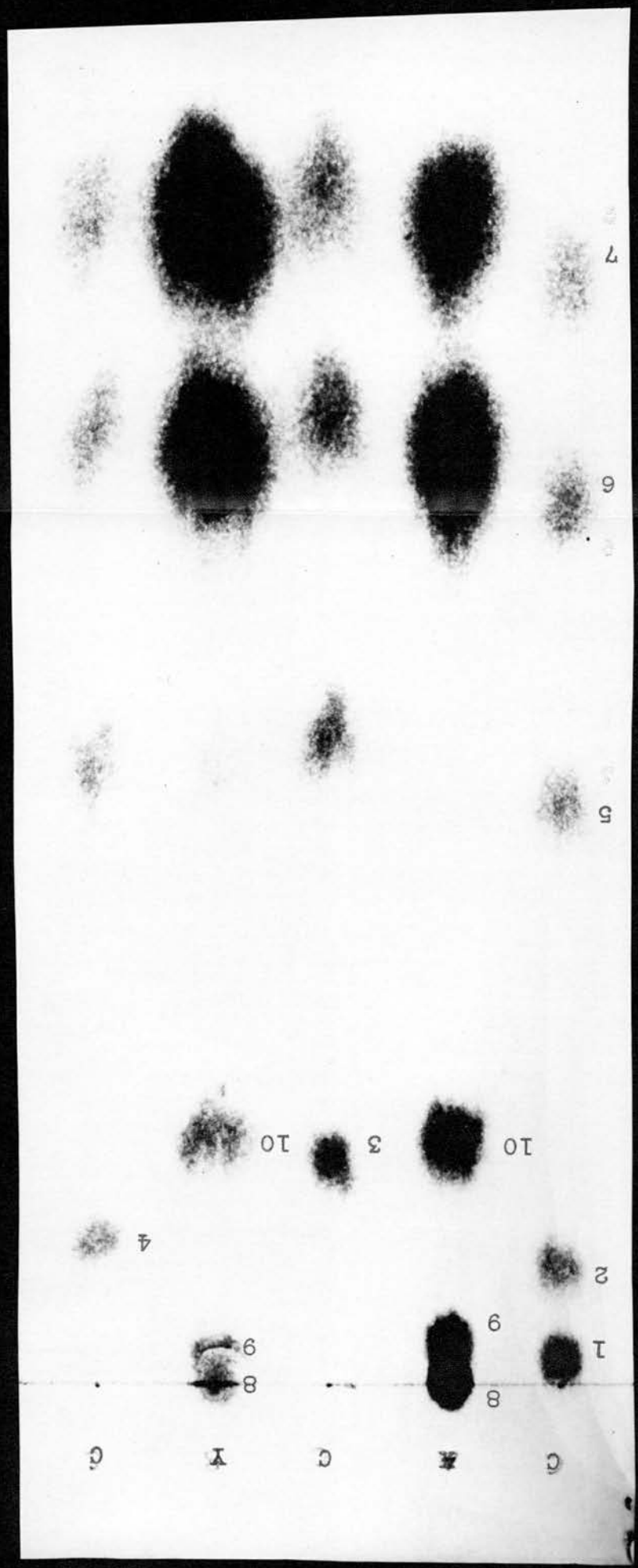
Wheat(40)

X 0·1N H_2SO_4 2 hr. 100°C

Y 0·2N H_2SO_4 2hr. 100°C

1-10 as above

C control sugars


G
A
C
B
G
1
2
3
4
5
6
7
8
9
10

G
A
C
B
G
1
2
3
4
5
6
7
8
9
10

PLATE IV

Plate IV. Partial hydrolysis products of cereal pentosan gums and hemicelluloses.

(Solvent, butanol:ethanol:water, 1% NH_3 by wt. in aq. phase)

A_1 rye(40) gum, 0·02N H_2SO_4 2hr. 100°C

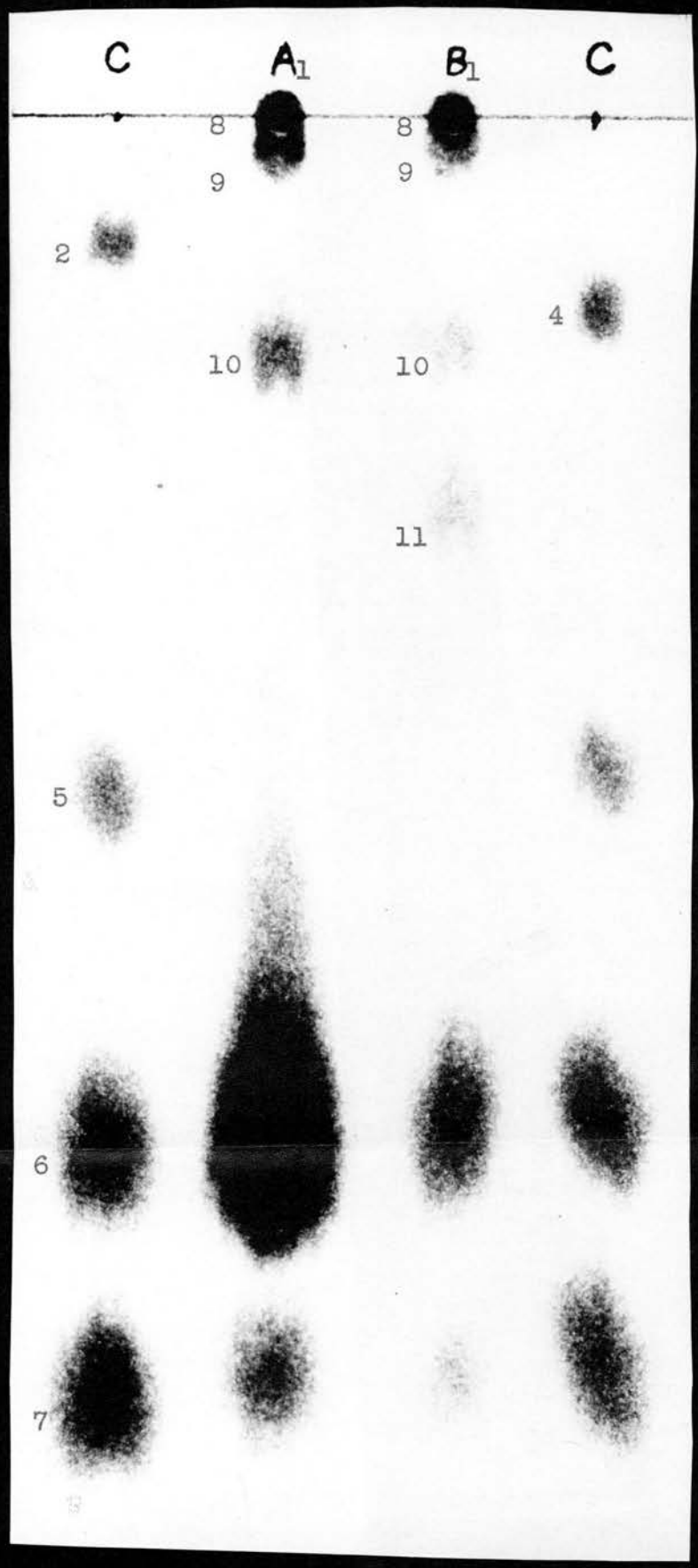
B_1 barley husk hem., 0·02N H_2SO_4 2hr. 100°C

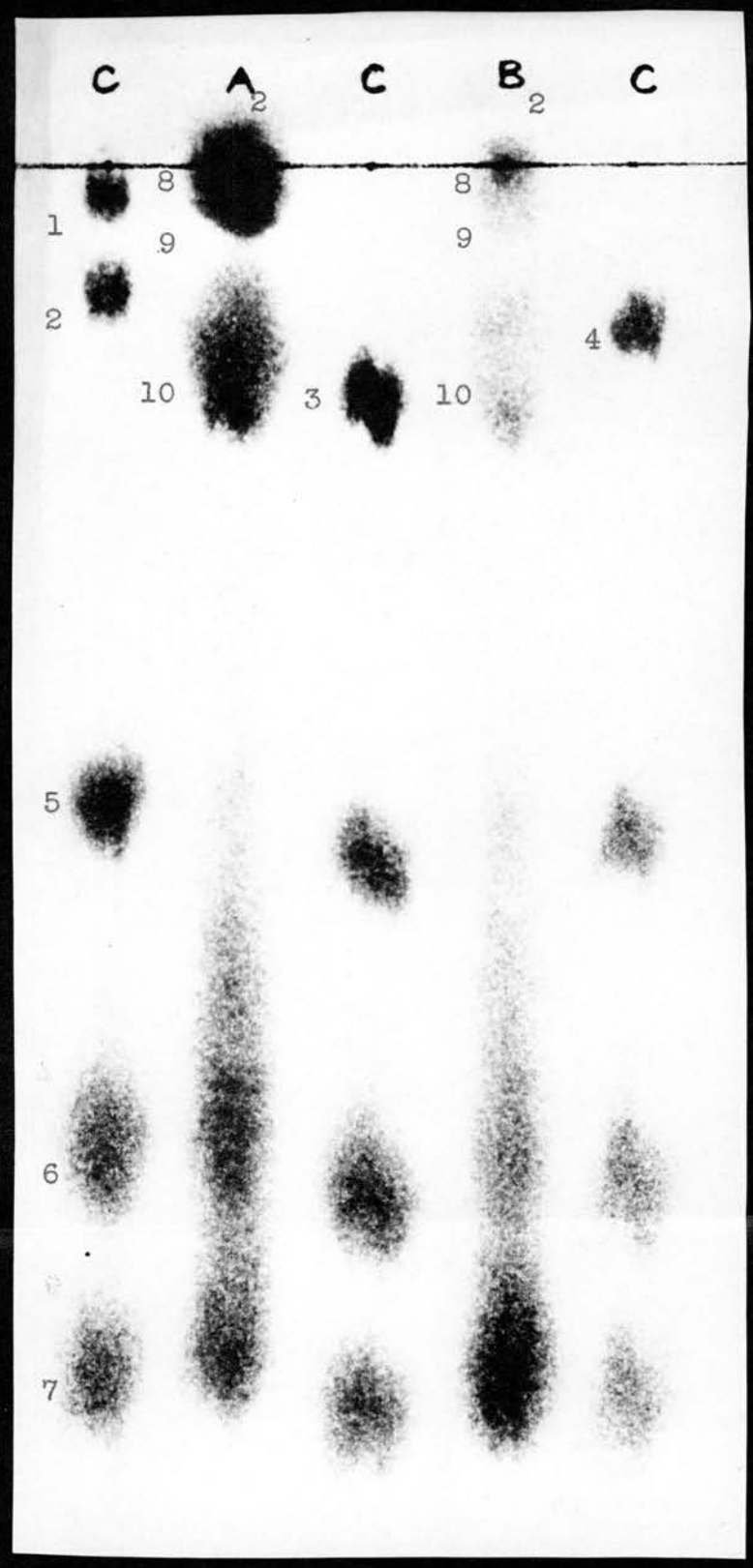
A_2 oat husk hem., 0·1N H_2SO_4 2hr. 100°C

B_2 oat husk hem., 0·2N H_2SO_4 2hr. 100°C

C control sugars

1 galacturonic acid
2 cellobiose
3 laminaribiose
4 maltose
5 glucose
6 arabinose
7 xylose
8 pentose oligosaccharides
9 xylotriose
10 xylobiose
11 pentose olgosaccharide


C
A1
B1
C
8
9
8
9
2
4
10
10
11
5
6
7


C
A2
C
B2
C
1
8
9
8
9
2
4
10
3
10
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6
7

the paper for analytical purposes. Hydrolysis with N H_2SO_4 for 3 hr. yielded only xylose, while in two completely separate sets of determinations iodimetric titration after hydrolysis, compared with that before hydrolysis, yielded titre ratios of 2.00 and 2.09 respectively. Such results obviously indicate the presence of a xylobiose, both from the wheat and from the rye gum. In this connection it is of interest to note that this substance, and the chromatographic spot occurring immediately above it, (R_F 0.07 in butanol:acetic acid, 0.004 in butanol:ethanol), have R_F values in butanol:acetic acid corresponding to those observed by Sórensen (83, 84) to represent xylobiose and xylotriose respectively. Prolonged chromatographic separation in butanol:acetic acid has resulted in the resolution of the material of a lesser mobility than the presumptive xylotriose into four further spots including one on the starting line. It is very likely that these represent a homologous series of xylose oligosaccharides since acid hydrolysis has resulted in the liberation of xylose alone.

DISCUSSION

General discussion:- The extensionsof the salt fractionation technique to various cereal gums has served to prove the general usefulness of the method beyond the isolation of β-glucosan from barley. A somewhat similar type of material has been obtained from oats and in this connection the report of the presence of lichenin in this grain by Morris (50) is of interest. This is especially so since structural studies on β-glucosan (5) have recently brought to light the presence of 1, 3 and 1, 4 β-linkages. The preparation of a pentosan (an arabo-xylan), apparently hexosan-free, from rye grain is particularly gratifying, since the search for such a substance was one of the primary objects of this work.

The modifications introduced into the original technique may be considered justifiable in view of the results obtained. Fractionation at constant temperature has largely succeeded in eliminating the very large spread in gum precipitation. Again, dialysis of the salt-saturated mother liquor prior to evaporation has resulted in

a much higher yield (approx. fivefold) of the final gum fraction, although it must be borne in mind that the barley samples used by Mackenzie, and these employed here, were different. Nevertheless, with the exception of that from maize, the very similar gross compositions of these mother liquor fractions, possessing as they do comparatively high araban contents, would appear to justify the present technique. The observation of Preece & Mackenzie (72) of the presence of an araban-containing, xylan-free, mother liquor fraction from barley was largely due, no doubt, to degradation during evaporation. Supporting evidence for this idea would appear to reside in the observation that the present mother liquor fractions contain (again with the exception of that from maize) about twice as much araban as was found to be present in Mackenzie's preparation.

The gum fractions obtained in this work may at present be better considered to represent type mixtures rather than entities, at least until such time as some rigid methods of further fractionation can be applied. This is especially

advisable in view of the fact that only one sample of each cereal has been here investigated. Thus, rye gums precipitating at 40 and 50% salt concentrations are not invariably composed of pentosan material alone, small amounts of hexosan having been identified in subsequent preparations. Also, viscosity figures and yields are liable to considerable fluctuation, depending both on the exact method of preparation and on the sample of grain employed. For this reason major arguments based on comparative gum viscosities have not been, and will not be, developed in this work. Where extract evaporation has been practised prior to gum precipitation specific viscosities for the rye gum fractions (Table II) are in the range 1.89 - 3.18. Omission of the evaporation step has produced gum viscosities of 11 - 12, admittedly from different rye samples, but these nevertheless probably represent gums which have undergone less degradation than the former. The latter preparations may correspond to those obtained from wheat by Meredith et al. (49) possessing very high viscosity and believed by them to be nearer to the native gum than anything formerly described. These workers

have also observed the presence of nitrogen in their gum preparations, but whether this is to be considered as an integral part of the molecule, or due to contaminating protein, is uncertain. Whatever may be the case it does not seem unlikely that the evaporation procedure will result in the breakdown of a nitrogen complex yielding a product, admittedly of lower viscosity, but perhaps even nearer to the native polysaccharide itself. While the presence of nitrogen has not been sought here it is interesting to note that the preparations of Preece & Mackenzie (72), made in substantially the same manner as these listed in Table II, were found to contain none. Similar wide variation in β-glucosan preparations (74) from different barley samples have been observed. Despite such fluctuations it is noticeable that no matter what the viscosity, rye pentosan possesses a specific rotation of ca. -135°, while barley β-glucosan has a value in the region of -12°. No pentosan has, until now, been obtained at 20% salt concentration, but at 30% small amounts of such material have been observed (72, 74) in some of the barley glucosan fractions, probably as a contaminant. Despite this,

it is obvious from the present work that precipitation of cereal extracts in general, with 30% ammonium sulphate, is not a measure of β-glucosan content as suggested by Preece & Mackenzie (73).

Effeciency of recovery:- In comparison with the unfractionated Fehling's precipitated gums (73) the method of salt fractionation results in rather low recoveries. In general, only some 50% of that recovered with Fehling's solution and acetone is obtainable by the ammonium sulphate fractionation. Such losses become even larger when it is remembered that Fehling's precipitation itself may result in no greater recovery than 85% of the gum treated. While it is true that the polysaccharides obtained in the present work are not directly comparable with those described by Preece & Mackenzie (73), being prepared from different samples of grain, the above losses have been repeatedly demonstrated on numerous occasions and this aspect of the work will be dealt with in Section V.

β-glucosan:- Earlier work (72, 74) has indicated the existence of β-glucosan molecules at various levels of molecular aggregation or of varying chain length, progressively smaller molecules (with pro-

gressively diminishing viscosities) being precipitated as the salt concentration increases. Such a decrease in molecular complexity (as measured by decrease in viscosity) has been more directly shown by the fractional precipitation of a solution of the anomalous 20 - 30 oat gum fraction, results being set out in Table VII. A gradation in viscosity is seen to exist from 22 to 28% salt concentrations. Also, the 22 and 28% fractions have viscosities approaching very closely those of the 20 and 30% fractions respectively.

The results in Table IV indicate that in wheat, rye, and barley, the glucosan content falls to a minimum at 40 - 50% salt concentration and thereafter rises. It seems likely that the glucosan precipitating at salt concentrations of 50% and upwards will consist wholly or partly of dextrinous material (α-glucosan). However, the task of detecting separately α-glucosan and β-glucosan, in a mixture of the two, is an extremely troublesome one. In this direction the action of α-amylase on some of the gum fractions is of interest, especially where those

TABLE VII

Viscosity Reduction of Oat β-glucosan with increasing Ammonium Sulphate Concentration required for Precipitation.

$(NH_4)_2SO_4$ precipitation level (%)	Recovery from mixture (%)	Specific Viscosity (0.5% soln., 25°C.)
20	-	1.24
22	69	1.25
24	11	0.77
26	9	0.63
28	6	0.50
30	-	0.59

gums containing glucosan material, and giving coloration with iodine, are concerned. The deep blue iodine coloration given by the 30% fractions from wheat and rye can be eliminated by α-amylase, the reaction being accompanied in the former case at least, by an increased negative rotation. The apparent presence of starch in these 30% fractions is peculiar since the gum solutions are normally very clear. In this connection the suggestion of the synthesis of hemicelluloses from starch precursors (18,58) might prove worthy of consideration, especially since O'Dwyer has been able to remove glucose residues from oak wood hemicelluloses by diastase, thus destroying the iodine coloration. Whether starch as such is present in these 30% gum fractions cannot be decided upon at present but if it is, its presence may be a function of the sticky nature of the pentosans, since no starch can normally be detected in the glucosan-rich 30% fractions from barley and oats. It is noteworthy that almost all subsequent 30% rye fractions have persisted in possessing a variable glucosan content (4 - 10%) and in giving a greenish to a deep

blue coloration with iodine. In view of the finding of dextrinous material in wheat gums by Perlin (64) particular interest is centred in the somewhat low negative rotations (ca. -100° as compared with ca. -135° in the case of rye pentosan) of the pentosan-rich fractions from wheat in the present investigations. ∝-amylase treatment of the 50% fraction from wheat has provided good evidence for the presence of dextrin, resulting as it has in the appearance of hexose-containing oligosaccharides, a marked change in specific rotation from -68° to -112°, and the removal of iodine-colouring material. Controls carried out using β-glucosan as substrate show complete absence of hexose oligosaccharides, thus indicating the source of the latter to lie in some non-β glucosan material, most probably starch or dextrins. Although ∝-amylase action has resulted in the removal of glucosan material, only one virtually complete removal (determined by chromatographic examination of the hydrolysis products of the residual polysaccharides) has been obtained. The fraction concerned, wheat gum 30%, shows a change of specific rotation from -110° to -134°, the

latter value being typical of hexosan-free pentosans prepared from rye. However, incubation at 50°C. has been found to remove glucosan material completely from a rye gum, although the rotational change was not determined.

On the basis of the above results it would appear that β-glucosan, if it does exist in the water-soluble polysaccharides of wheat and rye, must be present in minimal amount. Glucosan content of these gums is thus virtually wholly of the α-configuration, and is much more prevalent in wheat than in rye. The rotations of the maize gums are indicative of dextrinous contamination while in the barley and oat gums it would appear on similar grounds that β-glucosan persists throughout the salt fractions to a very great extent.

While it has not been possible to apply methods for the structural investigation of these polysaccharides, some degree of comparison of the different types has been achieved by hydrolytic techniques. Some success is evident (Table VI) in the partial acid hydrolysis of the barley and oat β-glucosans, three oligosaccharides and glucose being detected in each case, besides

higher molecular material. Two of these oligosaccharides correspond, in two solvent systems, to cellobiose and laminaribiose respectively. The presence of 1, 3 and 1, 4 β-linkages in barley β-glucosan has been established (5) and consequently similar linkages may exist in the oat polysaccharide.

Pentosan-rich fractions:- From what has been said it seems safe to assume that the glucosan present in essentially pentosan fractions may be of one, or both, of two types, namely β-glucosan and α-glucosan, the latter presumably being related to starch. Moreover, it is fairly certain that the β-glucosan represents a quite separate molecular species from the pentosan, existing merely as a contaminant in pentosan fractions. It is also highly likely that the dextrinous material is simiarly disposed although such fractions as those obtained from rye and wheat at 30% salt concentration may possibly represent special cases. The preparation of a hexosan-free pentosan has added much impetus to this work, ascertaining as it does the presence of such material in the rye grain, and indicating its probable existence throughout the

cereals investigated, to a greater or lesser extent. If one neglects the glucosan portions of the pentosan-rich gums, an apparently justifiable step, the xylan:araban ratios in each fraction can be calculated. Results are shown in Table VIII for all except the mother liquor fractions from rye, wheat and barley.

Where the 40% level is concerned the ratio is fairly constant, and were this the only criterion to be considered, the presence of a simple type of molecule of defined composition might be assumed in all three cereals. Again, if the wheat gums only are considered, showing as they do increasing solubility in water (as judged by ease of precipitation with ammonium sulphate) with decreasing xylan:araban ratio, there appears to be extremely strong support for the Perlin hypothesis of variable arabo-xylan composition (64). It must always be borne in mind, however, that the solubility relationships observed by the latter were based on the fractionation of the polysaccharide acetates with organic solvents. Even so the conclusions reached in the two cases are similar. It is interesting to compare the 40% fraction obtained

TABLE VIII

Ratio of Xylan to Araban in essentially Pentosan Gum Fractions
(Figures shown are calculated from the results of Table IV).

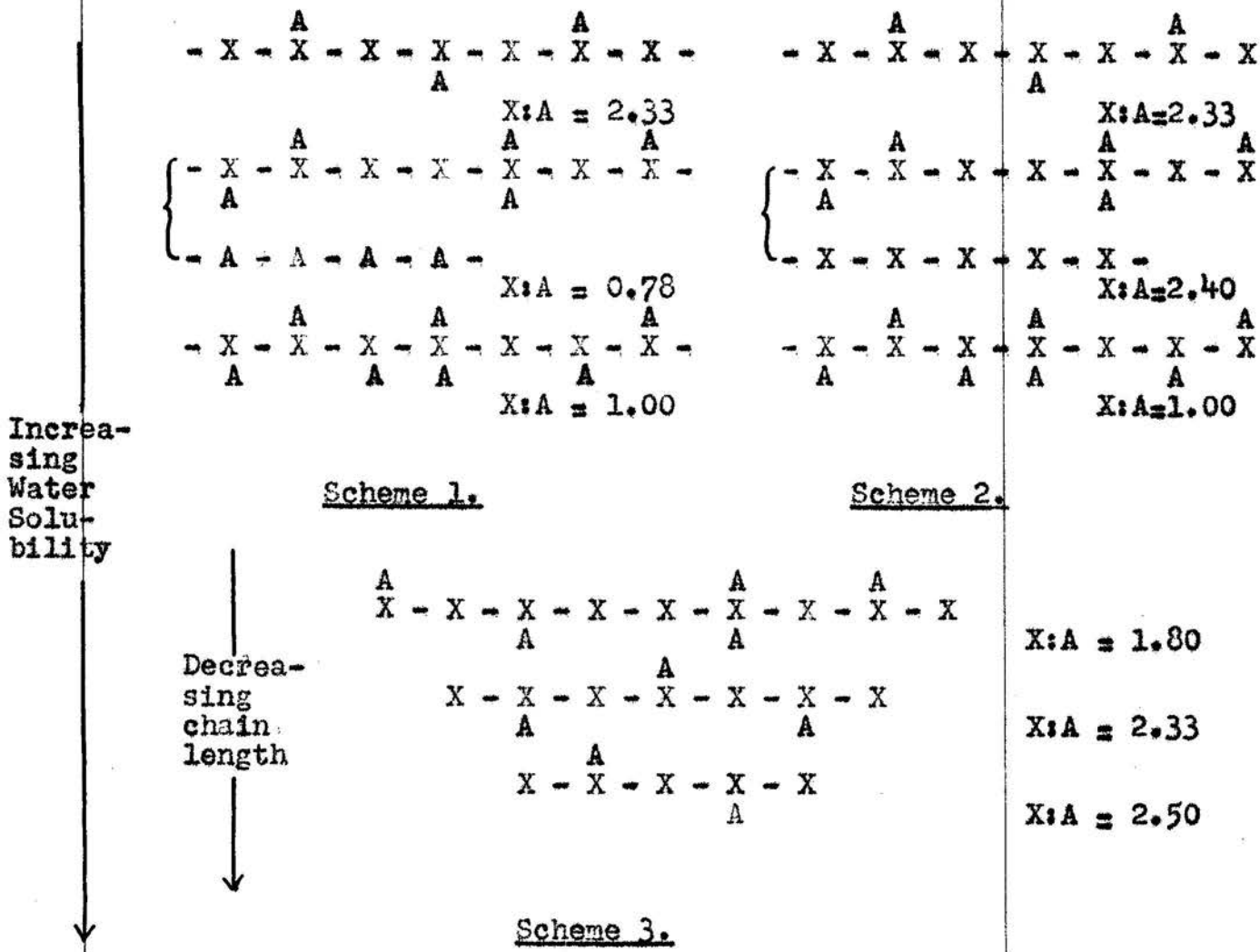
$(NH_4)_2SO_4$ precipitation Level (%)	Rye	Wheat	Barley
30	1.17	2.57	-
40	1.56	1.89	1.78
50	1.22	1.56	2.33
60	2.23	1.22	-
Saturation	-	-	6.39

from wheat in the present work, with certain fractions prepared by Perlin from wheat flour, which included a crude product possessing $[\alpha]_D^{25} = -110^\circ$ (1% gum in 0.5% H_2SO_4); glucosan = 3%, araban = 36.3%, xylan = 57.1%, and galactan = 3.6%. Further fractionation of this product by Perlin yielded two fractions, one of which had $[\alpha]_D^{25} = -108.7^\circ$, a trace of glucosan, 36.6% araban, 62.2% xylan, and 1% galactan. This, except for the presence of galactan is very similar to the 40% fraction from wheat, the latter having $[\alpha]_D^{15} = -100^\circ$ (0.5% gum in aqueous solution), glucosan = 6%, araban = 33%, and xylan = 61%. While the wheat gums conform to the plan outlined by Perlin, those from barley (72) actually behave in a reciprocal fashion, with the least soluble containing the most araban. In the case of the rye polysaccharides no definite trend can be detected. Insofar as these observations are concerned it is clear that the simple Perlin hypothesis falls short of a complete explanation.

However, in view of additional information, the presence of a certain proportion of the type of molecule envisaged by Perlin is more

than likely. It has been repeatedly observed that the removal of arabinose residues from the pentosan gums by acid hydrolysis results in the subsequent precipitation from solution of the modified polysaccharide. This much is in agreement with the conception of a xylan chain possessing side chains composed of arabinose residues, and therefore with the Perlin idea. In this case, fractions of greater solubility might be expected to contain a proportion of molecules of similar principal (xylan) chain length but with more or longer araban side chains, the gross composition being modified by the presence of other material. What this material might be cannot at the moment be ascertained. The possibility of the presence of "pure" araban in certain of the fractions should not be overlooked (Fig. II, scheme I) although extraction with boiling 80% ethanol has not resulted in any removal of araban from certain pentosan gum fractions.

Two further possibilities present themselves and seem worthy of consideration. Firstly, "pure" xylan of varying molecular dimensions or varying degrees of molecular aggregation might be



Scheme 1.

Scheme 2.

Scheme 3.

Figure II. A diagrammatic representation of the possible constitution of cereal pentosan fractions.

(X denotes xylose residue; A denotes arabinose residue).

distributed among the various fractions in such a way that the largest are to be found in the least soluble fractions. Larger amounts of shorter chains in the more water-soluble fractions might account for their higher xylan:araban ratios (Fig. II, scheme 2). It may be pointed out, however, that such a state of affairs is unlikely considering the inherent water insolubility of a xylan chain of the above type. Nevertheless, a slight modification involving a very small proportion of arabinose and/or uronic acid residues, might help to explain the observed facts. Alternatively, Perlin-type molecules composed of xylan chains of different lengths, shorter chain lengths possessing greater water solubility, although perhaps associated with fewer or shorter araban side chains, might provide the answer (Fig. II, scheme 3). Such a possibility has much to commend it on general grounds, suggesting as it does a greater degree of uniformity of organization, and it appears at present to be more worthy of acceptance than does either of the foregoing hypotheses. (Fig. II is a diagrammatic representation only, and the x:a ratios bear no relationship to values found for the

gums). It seems likely that similar considerations will apply where the small amounts of maize and oat pentosan are concerned although accurate assessment of the xylan:araban ratios in these cases is not easily possible owing to the very small quantities involved.

The water-soluble cereal pentosans are much more susceptible to acid hydrolysis than are the glucosans. Hydrolysis under relatively mild conditions results in the preferential removal of arabinose units; such an observation is in agreement with Bywater et al. (17) and thus supports the general molecular structure proposed by Perlin. Hydrolysis with acid of varying normality has lead to the accumulation of a xylobiose, a presumptive xylotriose, together with xylose oligosaccharides, arabinose, and xylose. Pentosans obtained from various cereal sources have been found to behave in identical fashion under such conditions.

Mother-liquor fractions:- These are non-precipitable by ammonium sulphate, a fact probably due either to their chemical nature, or to their molecular size, or both. In view of their

general properties they scarcely merit the term 'water-soluble gum'. They are characterized by their very similar compositions (with the exception of the maize fraction), possessing as they do relatively high araban content, a narrow range of specific rotation, -18° to +18° (a prepared malt fraction had $[\alpha]_D^{15} = +22°$), and very low viscosity. ∝-Amylase action (Table V) has demonstrated without doubt the presence of dextrinous material. Removal of iodine-colouring substances, decrease in positive rotation, and the identification of maltotriose, maltose, and glucose after enzyme incubation, is all indicative of dextrin removal. The constitution of the remainder of the individual fractions is uncertain. It may be suggested that the galactan and the bulk of the araban are in the form of 'pure' polysaccharides such as are found in association with pectin (38). However, the possibility of the presence of an arabo-galactan of the type described in larchwood by White (87) may be considered. The remainder of the araban together with the small amount of xylan will presumably exist as short chain arabo-xylan of the type discussed above.

The apparent absence in the water-soluble gums in general, and in the above mother liquor fractions in particular (assuming a relationship between the latter and the pectic materials), of uronic acid residues, is surprising, and even disturbing. In view of this, an investigation into uronic acid participation in cereal polysaccharides has been made, and is described in Section III.

Cereal relationships:- In respect of gum composition the five cereals investigated form a series the extremities of which are represented by rye and maize. The former is exceptionally rich in pentosan of high molecular complexity, β-glucosan contamination being small if it occurs at all. Wheat is also rich in pentosan although glucosan contamination, which appears to be mainly, if not wholly, dextrinous, is much more widespread than in rye. Pentosan content of the barley polysaccharides is much smaller but large amounts of uncontaminated β-glucosan are recoverable. In this case the spread in β-glucosan has rendered the preparation of a hexosan-free pentosan impossible so far. Dextrinous contamination occurs at the higher solubility levels. Barley would appear to provide

a link between rye and wheat on the one hand, and oats on the other. The solubility spread of β-glucosan in oats is greater than in barley and the pentosan content of the former cereal is obviously smaller. In maize the content of water-soluble pentosan is minimal, β-glucosan is apparently absent, and dextrinous contamination is great. It is doubtful indeed if there is more than a trace of 'true' water-soluble gum present in this cereal. Similarities and differences in chemical behaviour of the gums are not unrelated to the taxonomic positions of the cereals concerned, an observation found to apply to the simpler carbohydrates of cereal grains (45).

Finally, a few important industrial implications of the above findings bear mentioning. As pointed out in the introduction Pence et al. (62) have observed the effect of pentosan materials on the consistency of doughs in the baking industry. The extremely sticky nature of the wheat pentosans prepared in the present work is in agreement with this. Again, Clendenning & Wright (21) describe the interference of highly viscous wheat pentosans during the preparation of starch from this source.

This is similarly borne out by investigations described in this section. Moreover, the use of maize as a source of starch finds support here, since little pentosan is present. This being so, virtually no interference will be encountered during starch extraction.

THE FURTHER FRACTIONATION OF SOME POLYSACCHARIDE FRACTIONS

INTRODUCTION

The ammonium sulphate fractionation technique, while resulting in most valuable information, has not proved unequivocally the separate nature, or otherwise, of the hexosan and pentosan gums. Thus, although the work described in the previous section strongly suggests the separate existence of the two molecular types, it is felt that stronger confirmation should be sought. Moreover, the possible presence in pentosan fractions of araban and/or xylan of the type proposed by Isherwood (40) could perhaps be investigated more fully. In this connection special interest resides in the mother liquor fractions which possess very small xylan: araban ratios. The likelihood of the participation of free araban, or at least araban in a different form from that in arabo-xylan, in the composition of the latter fractions, has already been mentioned. Because of this, as well as for other reasons, the mother liquor fractions have been largely employed in this investigation. The application of some mainly physical methods of fractionation were applied and although the

results obtained are primarily of a negative nature they may serve as a starting point for future work.

EXPERIMENTAL

Fehling's precipitation:- A fraction precipitated at 40% ammonium sulphate, and obtained from rye, was made up in aqueous solution (ca. 1%) and treated with an equal volume of mixed Fehling's solution. Although no precipitate was formed, an apparent increase in viscosity was observed (cf. Preece & Ashworth, 71). Acetone was added to approximately 13% by volume whereupon a precipitate exhibiting extremely gelatinous properties was obtained (F_1). After centrifuging, the mother liquor was made up to 26% with respect to acetone to give a further precipitate (F_2), less gelatinous than the first. Precipitation of the mother liquor from this latter fraction, at 40% acetone, yielded a third precipitate (F_3). The copper complexes were decomposed with dilute HCl and the acid solutions treated with acetone to 60% whereupon the free polysaccharides were recovered. These were washed with 60% acetone and dried off with increasing concentrations of ethanol in the

normal manner. Acid solubility appeared to decrease from the gelatinous F_1 fraction to the somewhat fibrous F_3 one. Each of the three polysaccharide fractions was hydrolysed with N H_2SO_4 and the products examined chromatographically in butanol: acetic acid:water (40:10:50). In this way no apparent difference could be detected between the fractions in respect of relative amounts of sugars. No strictly quantitative investigation was undertaken.

Chromatography: A preliminary investigation into the possibility of obtaining chromatographic separation of some gum fractions has been made. Straightforward elution analysis was carried out with various solvents on columns (11 x 2.5 cm.) of Whatman cellulose powder packed either in water or 45% ethanol. Partition chromatography was performed employing celite as the inert support (columns 10 x 1 cm.).

Solvents used for elution analysis included ethanol, aqueous ethanol, and various mixtures of ethanol, acetone and water. It is felt that precise information about these is not justified by the results obtained. Various mother

liquor gum fractions were run using ca. 100 mg. in each case in 2-3 ml. aqueous solution. After application the solution was washed into the column with 5 ml. solvent prior to development. The eluate was collected in 3ml. fractions, carbohydrate being tested for by means of the Molisch reaction. In this way no obvious separation was observed in any case, the entire carbohydrate being recovered in a volume of about 10 ml. In one instance, where 16 ml. of eluate were found to contain the polysaccharide, the run was repeated collecting 2 ml. fractions over this range, evaporating each to dryness in vacuo, and hydrolysing in each case for 3 hr. with N H_2SO_4. After neutralizing etc., each fraction was examined by means of paper chromatography (butanol:acetic acid: water: Whatman No. 1 paper) but no qualitative distinction could be observed between the eight fractions. On a partition chromatogram employing butanol:ethanol:water (45:5:50) as solvent system, with 8 ml. stationary phase per. 10 ml. celite, no separation was obtained.

Electrophoresis: Electrophoretic treatment was applied to several mother liquor fractions

employing a paper electrophoresis apparatus modified from that described by Durrum (31), the general technique applied being as follows. Whatman No. 3MM. filter paper strips (ca. 44x6 cm.) were used as the supporting medium, with standard buffer solutions of sodium borate (0·002M & 0·02M, pH 11) and sodium acetate (0.003M & 0.03M, pH 4). Horizontal bands of polysaccharide solution (ca. 0.5% aqueous) were applied to a central line on the paper by means of a glass chromatographic pipette and dried at about 40°C. Great care was necessary during this operation since the polysaccharide, on drying, formed a film resistant to penetration by the buffer solution subsequently involved. Papers thus prepared were soaked to within about half an inch of the starting line on either side, placed in position in the apparatus with the two ends dipping into the solutions in the anode and cathode compartments respectively, and the system allowed to equilibrate for 15 min. A certain voltage was then applied and the run carried through for a specified time, after which the paper was removed, dried at 100°C. (5 min.), sprayed with aniline oxalate reagent, and, after

development at 100°C., the bands were located. This method of detection is not particularly successful since polysaccharides do not yield strong colorations. Also, the colour of a band is no criterion of its constituents, a pink band (characteristic of pentose sugars) possibly containing a considerable proportion of hexosan. Plate V represents an electrogram of a rye mother liquor fraction in 0.02M borate buffer for 3 hr. at 300 v. and a current of 1.5 ma.-2.0 ma. The band on the starting line is only faintly visible in the photograph. Observations on several fractions are made in Table IX. The more dilute buffers resulted in a lesser movement and more diffuse bands. Also, a field strength of more than 300 v. proved rather unsatisfactory, the bands possessing fronts which were far from straight. Further work was performed with borate and acetate buffers of molarities 0.02 and 0.03 respectively, at a field strength of 300 v. (current ≈1 - 2 ma.).

For the purpose of investigating the constitution of separated bands the following procedure was adopted. Polysaccharide solution was

TABLE IX

Observations on the Electrophoretic Treatment of some Polysaccharide Fractions

Fraction*	Volts	M-amps	Time (hr.)	Buffer soln.	To anode χ	To cathode χ	Stationary χ
Rye	250	1·5	1	borate (0·02M)	-	brown pink	-
"	500	2·5-3	1	"	-	brown pink	-
"	400	2·0-2	2	"	-	Brown pink	pink
"	400	2·0-3·0	7	"	-	brown pink	pink
"	400	0·5	3½	acetate (0·003)	-	brown	pink
Barley	400	2·0-3·0	6	borate (0·02)	-	Ø	pink
Wheat	300	1·0-2·0	4	"	-	brown	pink
"	300	-	2½	borate (0·002)	-	brown	pink
"	300	-	2½	acetate (0·03)	-	brown	-
Oats	300	-	3	borate (0·02)	pink	-	-
"	300	1·0-2·0	2½	"	pink (brown?)	brown	pink
"	300	-	2	(acetate) (0·003)	-	pink	brown
"	300	1·0-2·0	10	(borate) (0·02)	brown pink	pink	pink
Maize	300	1·0-2·0	5	"	Ø	brown brown	

* Refers to mother liquor fractions.
χ Refers to colour of band after development.
Ø Band visible but of uncertain colour.

PLATE V

Plate V. Electrophoresis of gum mother liquor fractions.

A- rye mother liquor fraction(see Table X)

1 stationary band, mainly arabo-galactan

2 band to cathode

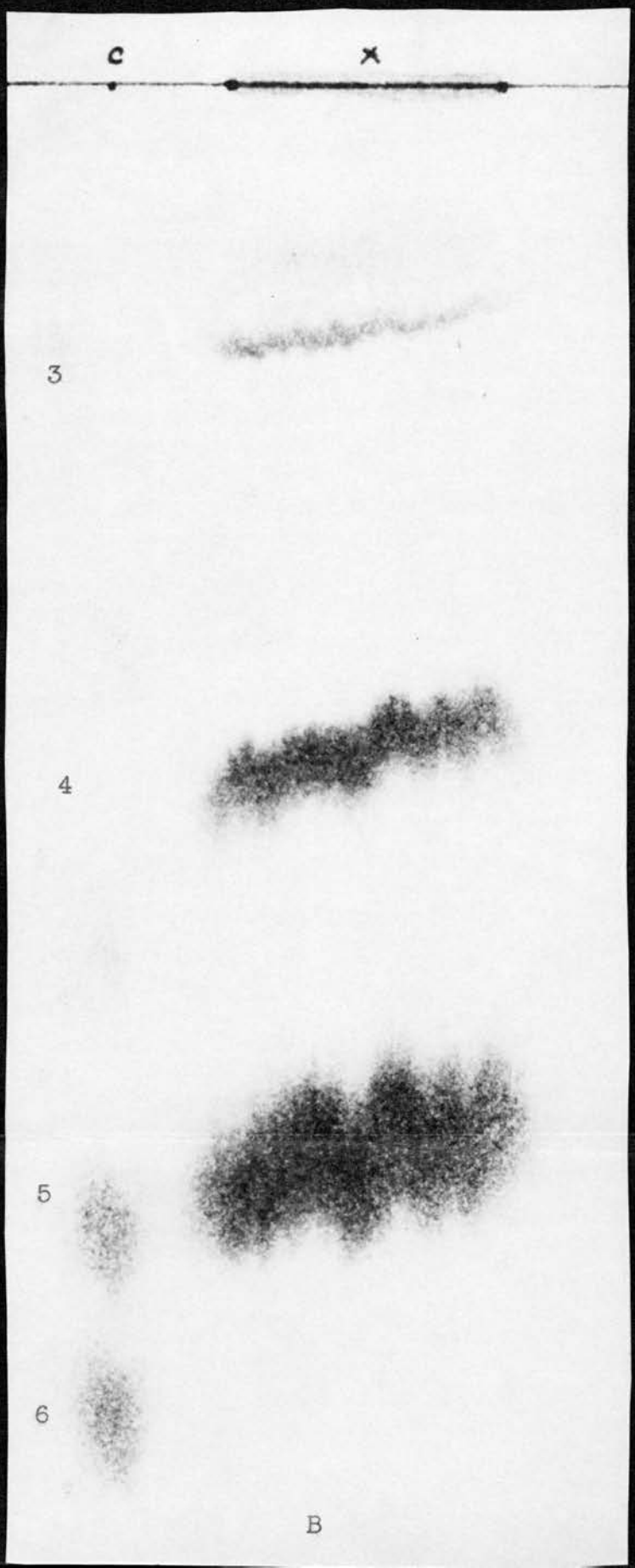
B- hydrolysis products of a separated band from an oat mother liquor fraction(see Table X)

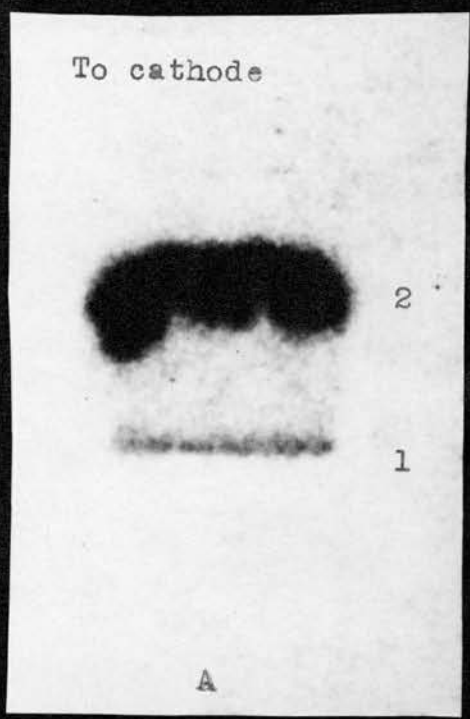
3 uronide-containing oligosaccharides

4 galactose

5 arabinose

6 xylose


C
X
3
4
5
6
B


To cathode
2
1
A

applied in bands as above but on this occasion together with control spots at each side. After running, and drying, longitudinal strips were cut, sprayed, and the various bands located. Bands were cut from duplicate electrograms, transferred to 50 ml. conical flasks, each containing 5 ml. distilled water, and stood at 40°C. with occasional shaking. After 30 min. the extracts were filtered off and the process repeated with a further 5 ml. water. The combined extracts in each case were collected in 100 ml. round bottom flasks, 2 - 2½ ml. 5N H_2SO_4 added (final normality = ca.1), and hydrolysis was carried out under reflux for 3 hr. Hydrolysates were treated in the normal manner and chromatographed on Whatman No. 1 paper in butanol: acetic acid:water. Results are shown in Table X and the chromatogram of one fraction from an oat mother liquor polysaccharide preparation is shown in Plate V.

In an attempt to investigate the oat mother liquor preparation further, two bands, one migrating to the anode and one stationary (0.03M acetate buffer, Table X), were eluted and rerun in borate of molarity 0.02 for 4 hr. at 300 v. and

TABLE X

Chromatographic Investigation of Electrophetically-Separated Polysaccharide Fractions (after acid hydrolysis)

Fraction*	Treatment	Band χ	Sugars Identified			
			Galactose	Glucose	Arabinose	Xylose
Rye (see Plate V)	300v.,1·5-2ma.,3hr. borate (0.02M)	brown (to cathode)	+	+++	++	++
		pink (stationary)	+	±	+	±
Oats ≠	300v.,1·5-2ma.,3hr. borate (0.02M)	(to ≠ cathode)	++	+++	+++	+
		pink (to anode	++	-	+++	-
		pink (stationary)	++	±	+++	-
" ≠	300v.,2-3ma.,3hr. acetate (0.03M)	(to ≠ cathode)	+++	+++	+++	+
		pink(to** anode)	+++	-	+++	-
		pink (stationary)	++	-	+++	-

* Mother liquor fractions
χ Colour of band after development
≠ Colour uncertain
≠ Oligosaccharides present (see discussion)
+++ Major component
++ Minor "
\+ Trace.
± Uncertain presence.
\- Apparently absent.

** See Plate V.

1 - 2 ma. In each case it was found that virtually the entire fraction migrated to the cathode, with traces to the anode and on the starting line.

DISCUSSION

If the polysaccharides treated do indeed consist of mixtures it is fairly obvious that the very similar nature of the components is responsible for the difficulties involved in further separation. Although the techniques applied have not been attended by unqualified success they might possibly serve as a useful basis for future investigation.

As regards the Fehling's precipitation it is of interest to note that, so far at least, no precipitation of a soluble gum of the type concerned here has been obtained in the absence of acetone. The reason for this is not clear since certain other polysaccharides (e.g. some hemicelluloses) are frequently recoverable as the copper complex in this way. The fractions obtained by the Fehling's solution-acetone treatment of the rye 40% fraction do not apparently differ in respect of sugar unit composition. However, there was an obvious difference where the appearance of the three was concerned, both in the form of copper complexes and free polysaccharides. Whether

such differences are real, or whether they arise in the course of preparation, is uncertain.

Chromatographic analysis, insofar as it has been pursued, has yielded no positive information beyond ascertaining that certain polysaccharides can be applied to, and recovered from, a column. It is not inconceivable that separation might be effected by the use of suitable solvent systems.

The third approach, namely electrophoresis, has, rather surprisingly perhaps, resulted in the most positive and interesting information. Electrophoretic runs have been confined to the mother liquor fractions, these offering fewer experimental difficulties; exhibiting as they do ready water-solubility and low viscosity in solution. The most intriguing result has undoubtedly been the separation of an arabo-galactan fraction from the oat mother liquor preparation. While this may be regarded as an advance in our knowledge of this polysaccharide preparation it has not by any means ascertained the occurrence of arabinose and galactose residues in one molecular species; the existence of free araban and galactan must be considered

as an alternative possibility. Even so, the separation from glucose and xylose residues is gratifying.

It has already been shown that the glucosan portion of the mother liquor preparations consists at least mainly of dextrinous material and it is also highly likely that arabo-xylan molecules of similar structure to that of the higher-molecular gums, but of shorter chain length, and hence higher solubility, will also be present. Unless it be assumed that the main xylan chains carry side chains, each consisting of more than one arabinose residue, the theoretical minimum for the xylan:araban ratio would appear to be 0·5. In the present instance, where the mother liquor fractions are concerned, ratios of 0·17 - 0.30 (excluding the maize product) are obtained, indicating araban participation in some other form.

Although this study is of a preliminary nature several points arise from Table X. First of all, bands rich in glucosan possess virtually the total xylan content of the original polysaccharide preparation; furthermore, bands containing these sugar units exhibit predominant

migration to the cathode at both pH values (4 & 11) considered. Araban and galactan show a similar association, either with or without glucosan or xylan. In the latter case the arabo-galactan fractions exhibit migration to the anode or none at all. The oligosaccharides mentioned in Table X as being detectable in the oat fractions on hydrolysis, appear to consist, at least partly, of uronide material (Dische reation, 29). Without drawing any conclusions as to the mode of participation of this in the mother liquor preparation as a whole, the oligosaccharides do seem to be concentrated in certain bands. Thus, in borate buffer they are predominant in the band migrating to the cathode, while in acetate they are very largely concentrated in the band moving to the anode (only one of these is visible in Plate V), very minute amounts being visible in the others, especially in the stationary band in acetate buffer where they are virtually absent.

The theory on which these separations are based is at present unknown. Initially it was supposed that borate complexes might contribute something towards the movement but somewhat similar

behaviour in acetate buffer would appear to invalidate such a theory. Northcote, in a recent publication (55), has noted the migration of neutral polysaccharides to the anode in borate buffer pH 9·2 and indicates the necessity of borate for good separation.

To sum up, arabo-xylan and glucosan exhibit a marked degree of electrophoretic association irrespective of pH. Arabo-galactan appears to possess a greater pH dependency, particularly that band containing the greatest proportion of uronide. The virtual absence of uronide in one of these bands is indicative of the occurrence of two types of this arabo-galactan although it is by no means certain that mixed molecules are involved. There is good evidence, however, for the occurrence of araban in two forms. Such observations show the complexity of these preparations, a state of affairs which may prove to be much more prevalent than at first suspected.

THE ESTIMATION OF URONIC ACIDS IN THE PRESENCE OF EXCESS SUGARS AND ITS APPLICATION TO THE CEREAL POLYSACCHARIDES

INTRODUCTION

The continued non-appearance of uronic acids in acid hydrolysates of the cereal gums presents a difficult problem. It has been ascertained that uronic acids are not lost during the normal chromatographic procedures and it would subsequently appear that such residues are either absent from these polysaccharides or that they occur only in trace amounts. It should be pointed out that where uronic acids have been specifically sought (71, 64) only very small amounts, if any, have been reported.

The estimation of uronic acids in admixture with a large excess of true sugars represents a real problem. Decarboxylation procedures (24, 51) tend to become inaccurate under such circumstances since non-uronide carbohydrates give rise to indeterminate amounts of carbon dioxide. Photometric methods are similarly hampered due to the colorations given by true sugars. Carbohydrates,

when treated with concentrated mineral acids, yield compounds which give colorations with organic compounds such as diphenylamine, carbazole, indole, and substances possessing sulphydryl groups. Some of these reactions are given by different groups of sugars and even individual sugars of the same group may show different absorption spectra etc. By selecting appropriate acid concentration, and time and temperature of acid treatment, certain of these reactions have been made more specific (26, 27, 28). A specific color reaction for uronic acids (29), based on the principle employed by Dische for the microanalysis of sugars (25), has also been reported.

The work described below involves an adaptation of the Dische method (29) for the purpose of determining small uronide contents of polysaccharides. For the sake of comparison, a few decarboxylation experiments have been performed by the procedure of Dickson et al. (24).

EXPERIMENTAL

Decarboxylation:- The apparatus was exactly similar to that described by Dickson et al. (24) as modified from Nanji et al. (51). After each deter-

mination it was dismantled and cleaned with reagents in the following order; hot dilute HCl, tap water, chromic acid, tap water, distilled water, and finally acetone, prior to drying off at 100°C. CO_2-free distilled water was used throughout the experimental procedure. Decarboxylations were carried out in duplicate, for 5 hr. in each case, in 12% HCl at a temperature maintained between the limits of 140°C. and 145°C. Blank runs in the airtight apparatus yielded results so small as to be completely negligible in the final calculations. Several runs were made with known weights of galacturonic acid and glucuronolactone, guaranteed 99% and 95-99% pure respectively (L.Light & Co.Ltd.). The results of these determinations are summarised in Table XI.

In view of the probable existence of very low uronide contents of the polysaccharides it was necessary to employ 0·3-0·5g. quantities of the latter for analysis. Such weights were only available in a limited number of cases, three separate determinations being made (Table XII).

Colorimetric methods:- The semi-micro method for uronic acid estimation (Dische, 29) involves the

TABLE XI

Decarboxylation of known Uronic Acids

Sample	Wt. taken (g)	CO_2 found (g)	%CO_2 found	%CO_2* (theory)	%Recovery
Galacturonic acid	0.202	0.043	21.3	22.7	93.8
"	0.130	0.029	22.3	22.7	98.2
Glucurono-lactone	0.126	0.031	24.6	25.0	98.4
"	0.160	0.037	23.1	25.0	92.4
"	0.240	0.055	22.9	25.0	91.6

*Taking uronic acid samples as 100% pure.

TABLE XII

Uronide contents of some Polysaccharide Fractions by Decarboxylation

Fraction*	Wt. taken$^{\chi}$ (g.)	Wt. of CO_2 (g.)	%CO_2	%Anhydrouronic acid
Barley (20)	0.200	0.0000	0.00	0
Wheat(mother liquor)	0.374	0.0023	0.62	2.5
Oats (mother liquor)	0.398	0.0050	1.26	5.0

* Refers to method of preparation (see Table IV)
$^{\chi}$ Dry ash-free polysaccharide.

use of carbazole as chromogenic agent. Interference from true sugars varies according to the types involved and to their concentrations. Such interference is very significant in the case of cereal polysaccharides and provision has been made to allow for it. It was decided to carry out all colour reactions after preliminary hydrolysis of the polysaccharides thus enabling more direct comparison to be made with control sugar solutions of known composition. A modification of the original Dische method (30) enables distinction to be made between galacturonic and glucuronic acids and this has also been employed in the work described below. As in the original publication (30), the first method, that for the estimation of total uronic acid, will be referred to as the 100°C. method, whereas the second, the method of distinction, will be known as the 60°C. method. Some slight modifications have been introduced to provide a means for the elucidation of the problem on hand.

Scrupulous cleanliness is absolutely essential where such delicate techniques are involved and all glass apparatus was accordingly cleaned with chromic acid followed by thorough rinsing with tap

water and distilled water, in that order, prior to drying off in an oven at 100°C.

100°C. method:- 1ml. portions of the solutions under investigation are measured into Pyrex glass tubes immersed in ice-water. 6ml. of conc. H_2SO_4 (a suitable commercial sample, 98%w/v) are added in each case and the contents of the tubes thoroughly mixed by shaking, the temperature being kept down by re-immersion in the ice bath. The tubes are then heated for 20 min. in a boiling water bath and immediately cooled in tap water. 0·2ml. of a 0·1% ethanolic solution of carbazole (a commercial sample, twice recrystallized from benzene; m.p.243-244°C., m.p. of acetate, 182-183°C.) is added and the solutions thoroughly mixed. The pink colour found to develop in the presence of uronic acids increases in intensity over an initial 2 hr. period and then remains substantially constant for a further hour. Colours given by true sugars increase slowly in intensity over this 2 hr. period, and indefinitely thereafter, following a rapid initial development. Readings were made in duplicate, 2 hr. after the addition of the carbazole, in an EEL photoelectric colorimeter

(Evans Electroselenium Ltd.) in matched tubes (1·5cm. diam.) using a green filter No. 624, transmitting light of maximum wavelength 520mμ, and against suitable blanks.

Effect of carbazole on carbohydrate absorption curves:- Absorption curves were plotted from 450-570mμ for various mixtures of sugars with or without galacturonic acid; after heating with conc. H_2SO_4 for 20 min., both at zero time and after standing for 2 hr.; after the addition of carbazole, at zero time and after standing for 2 hr. These curves are shown in Fig. III.

Variation of optical density with sugar concentration:- Owing to the high colorations given by non-uronic carbohydrates under the above conditions it has become necessary to investigate this relationship. For this purpose glucose (a commercial sample), arabinose (British Drug Houses), and xylose (L.Light & Co.Ltd.) were employed with no treatment other than heating to constant weight at 90°C. Standardisation curves for glucose and pentose (xylose: arabinose, 7:3) are given in Fig. IV. Readings made with or without prior heating of the sugars with dilute H_2SO_4 were

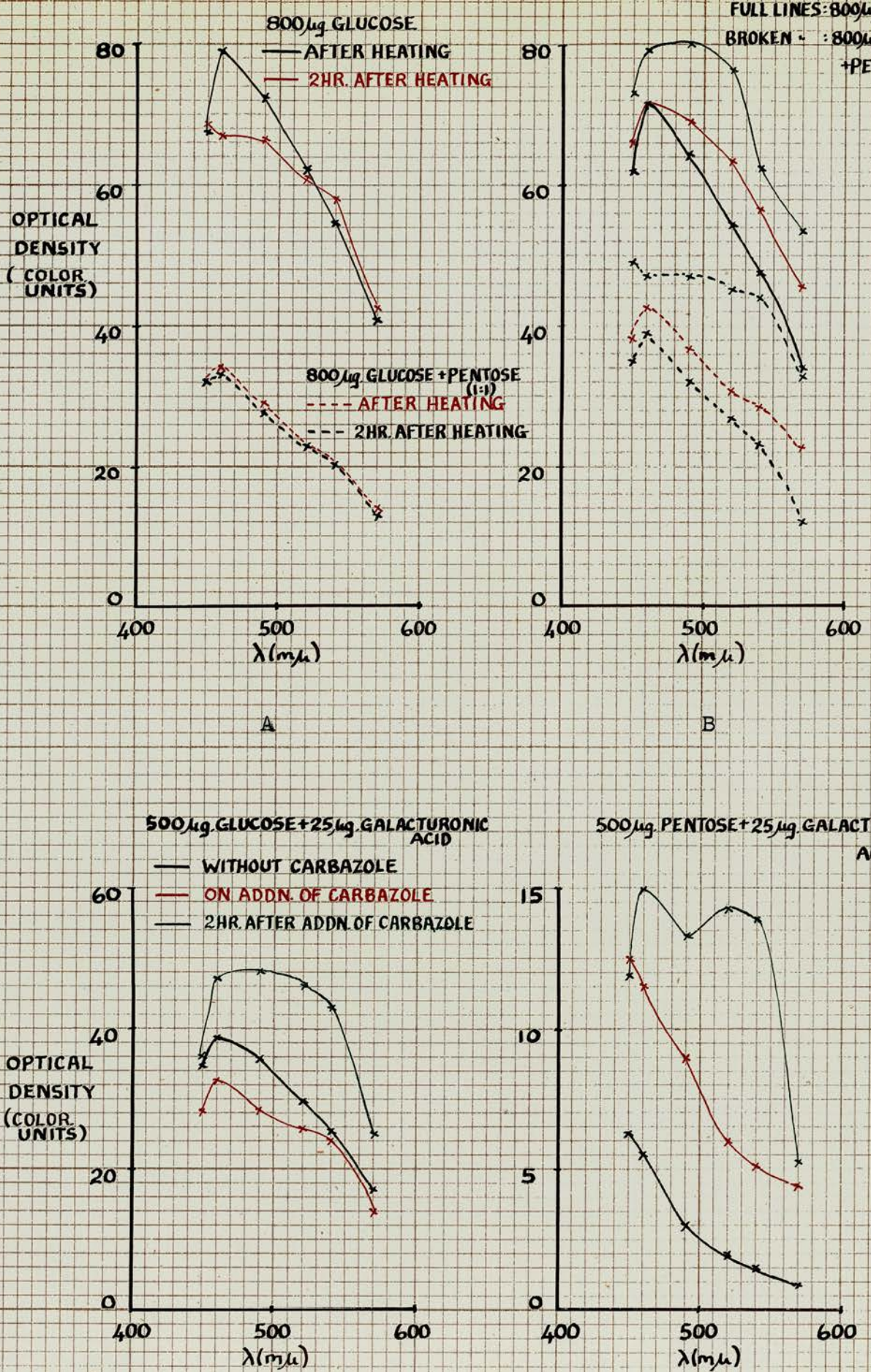
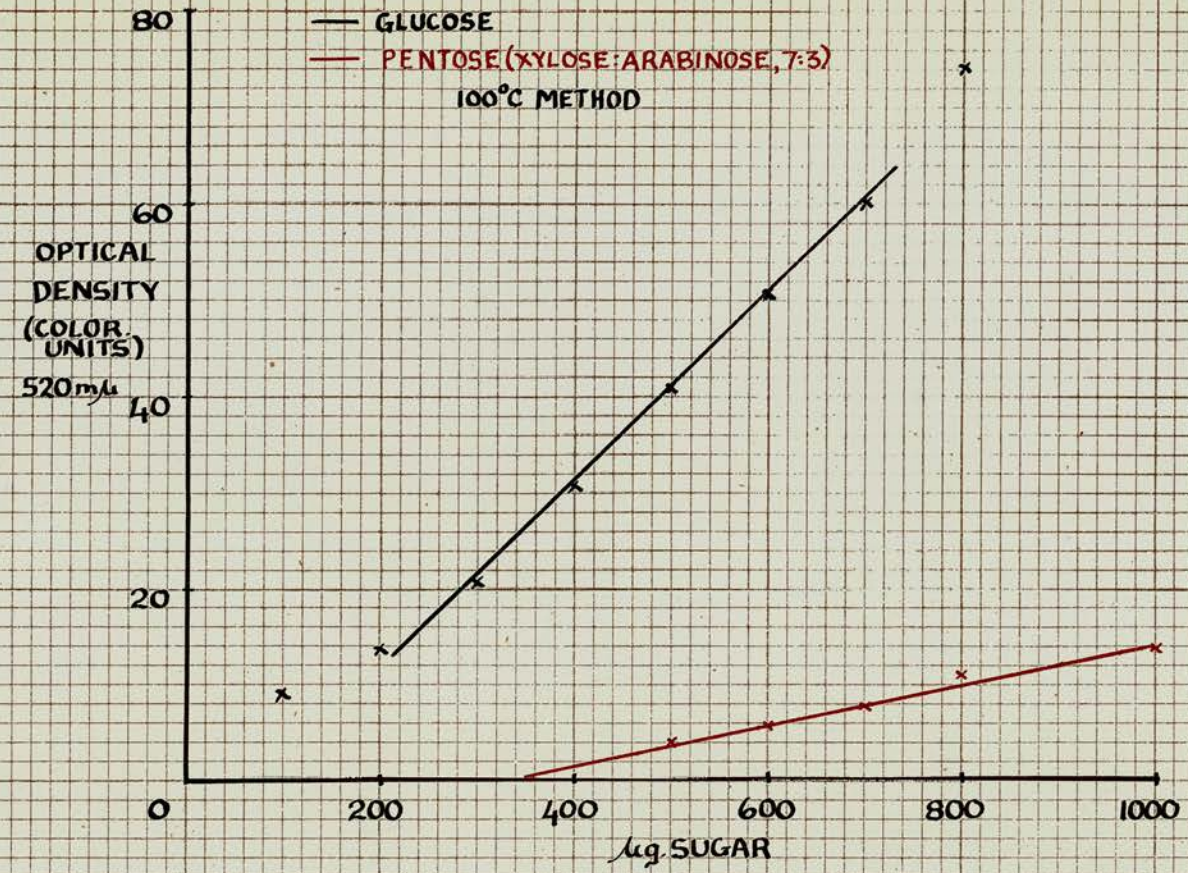


Figure III. Effect of carbazole on carbohydrate absorption curves. (Colours of curves in B & D correspond to those in C)



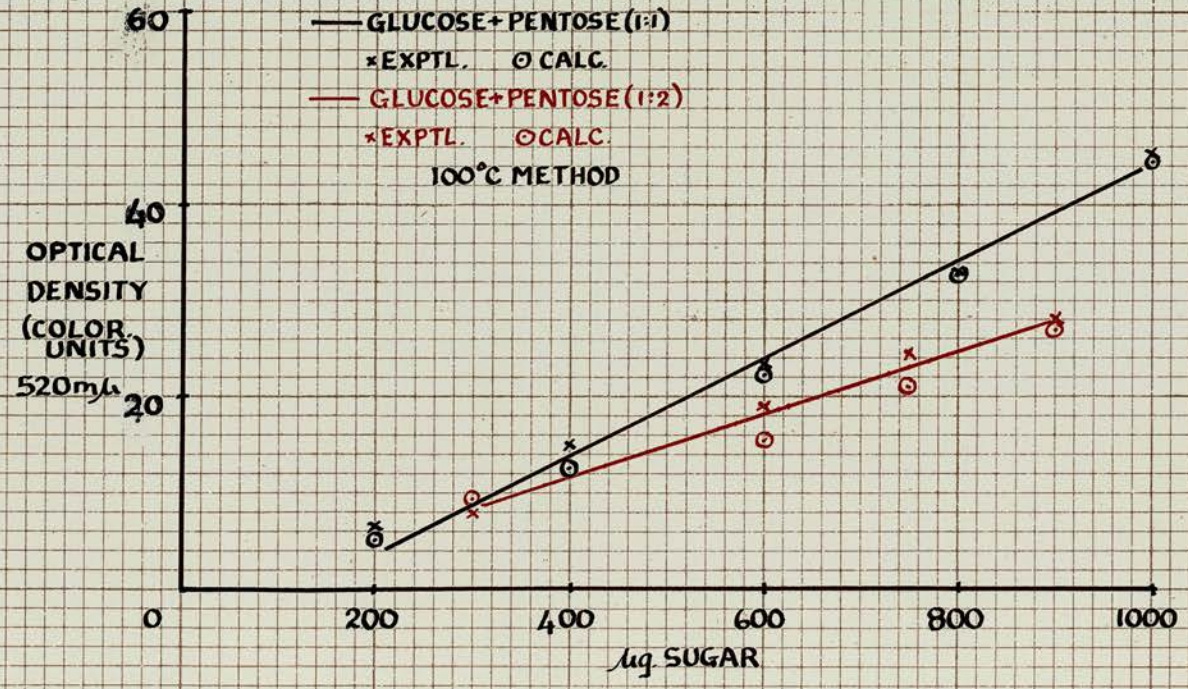


Figure IV. Variation of optical density with concentration for true sugars.

found to be very similar. However, it has been found that the intensity varies somewhat from one determination to another, this variation amounting to ±5% of the curves shown in Fig. IV. Duplicates in one and the same determination exhibit a somewhat lesser variation.

Standardisation curves for glucose/pentose mixtures are also shown in Fig. IV, the experimentally obtained curves being compared with those constructed from the separate curves for the component sugars.

Variation of optical density with galacturonic acid concentration:- A standardisation curve for galacturonic acid is shown in Fig. V, little difference being noted where previous heating with dilute acid has been employed. Intensities for galacturonic and glucuronic acids were virtually the same. Absorption curves for the two acids, exhibiting maxima at 520mμ, are also presented in Fig. V.

Estimation of uronic acids in the presence of excess sugars:- Table XIII contains data obtained on the recovery of uronic acids from sugar solutions. Figures are shown for mixtures both with and without previous acid treatment, uronic acids being estimated

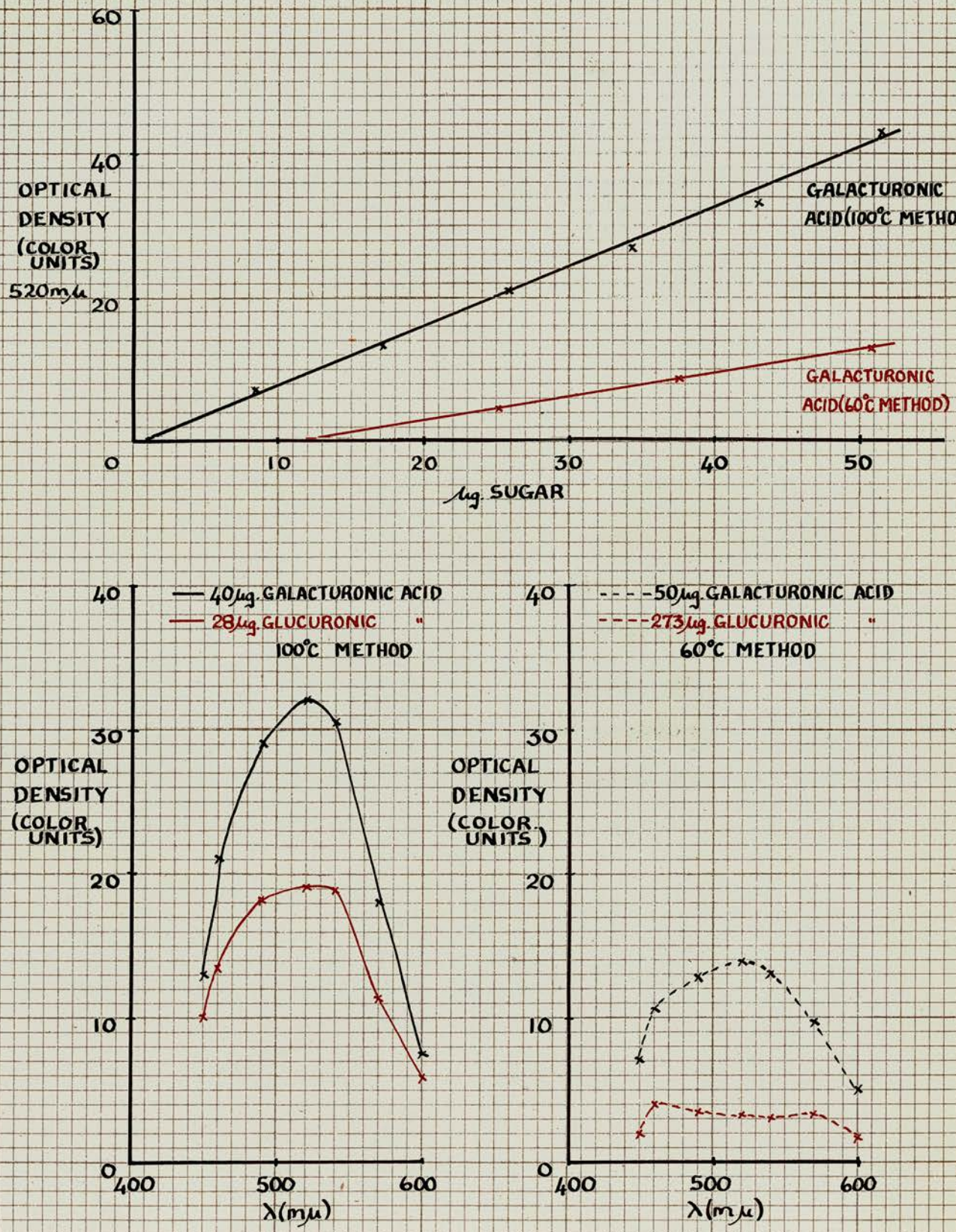


Figure V. Variation of optical density with concentration of uronic acid, and absorption curves for uronic acids.

TABLE XIII

Determination of Uronic Acids when present in an Excess of true Sugars

γg. Glucose*	γg. Pentose*	γg. Uronic* acid	Color. rdg. (total)	Color. rdg. (sugars)	Color.χ rdg. (uronic acid)	γg. Uronicχ acid (found)	%Recovery
-	1000 ∠	22	28.4	13.4	15.0	19.0	86
-	800	17	23.8	9.5	14.0	18.0	106
-	500	17	18.4	3.5	14.9	18.0	106
300	300	26	43.0	22.0	21.0	26.0	100
400	200	26	52.7	31.5	21.2	26.0	100
500 ∠	-	22	58.0	41.0	17.0	21.0	95
500	-	26	58.2	41.0	17.2	21.0	81
300	-	9	28.5	22.0	6.5	9.0	100

* γg.ml. of solution used for analysis.

χ Obtained from: color.rdg.(total) minus color.rdg.(sugars); to nearest unit.

∠ Employing preliminary acid treatment.

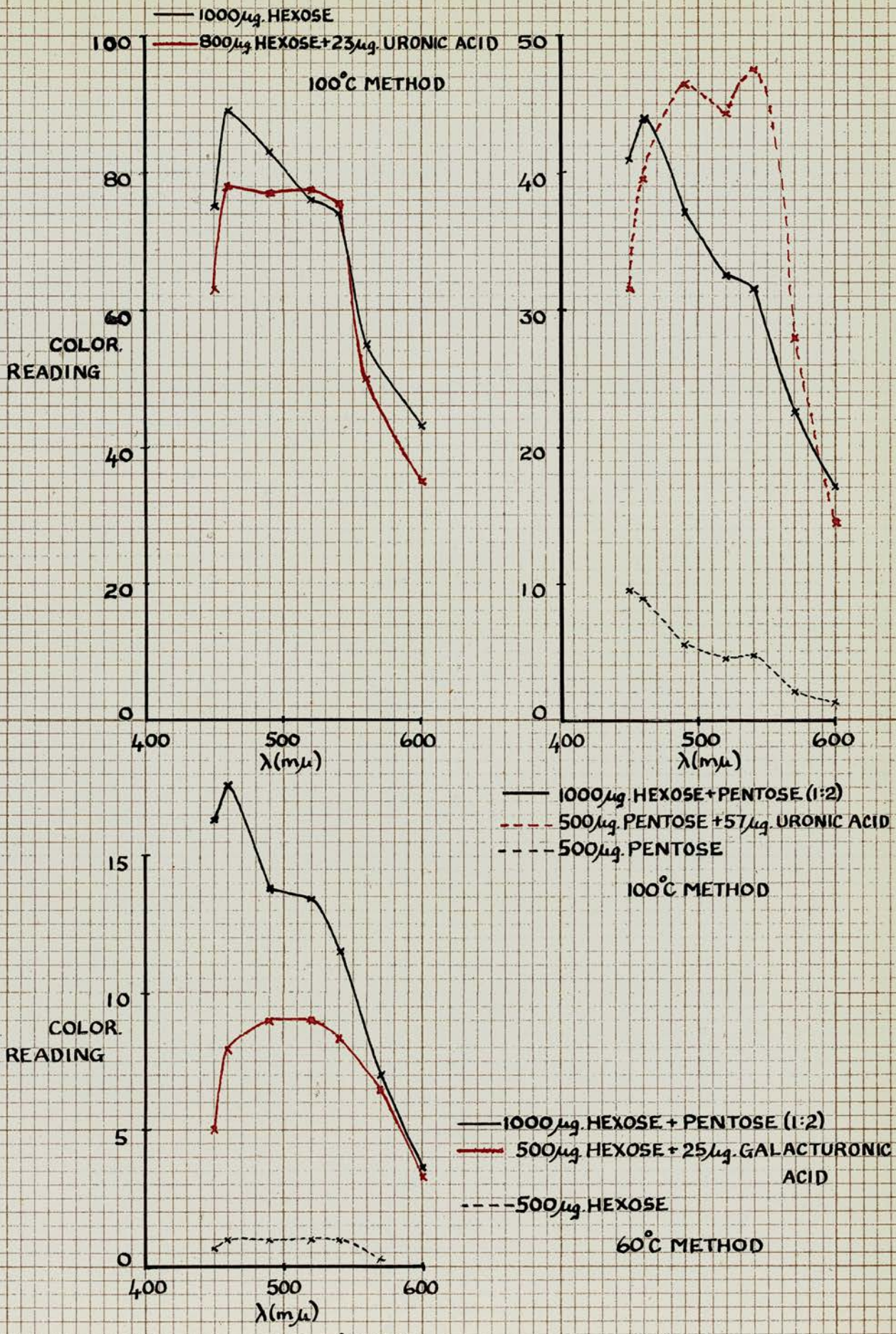


Figure VI. Absorption curves for sugar, and sugar/uronic acid known mixtures.

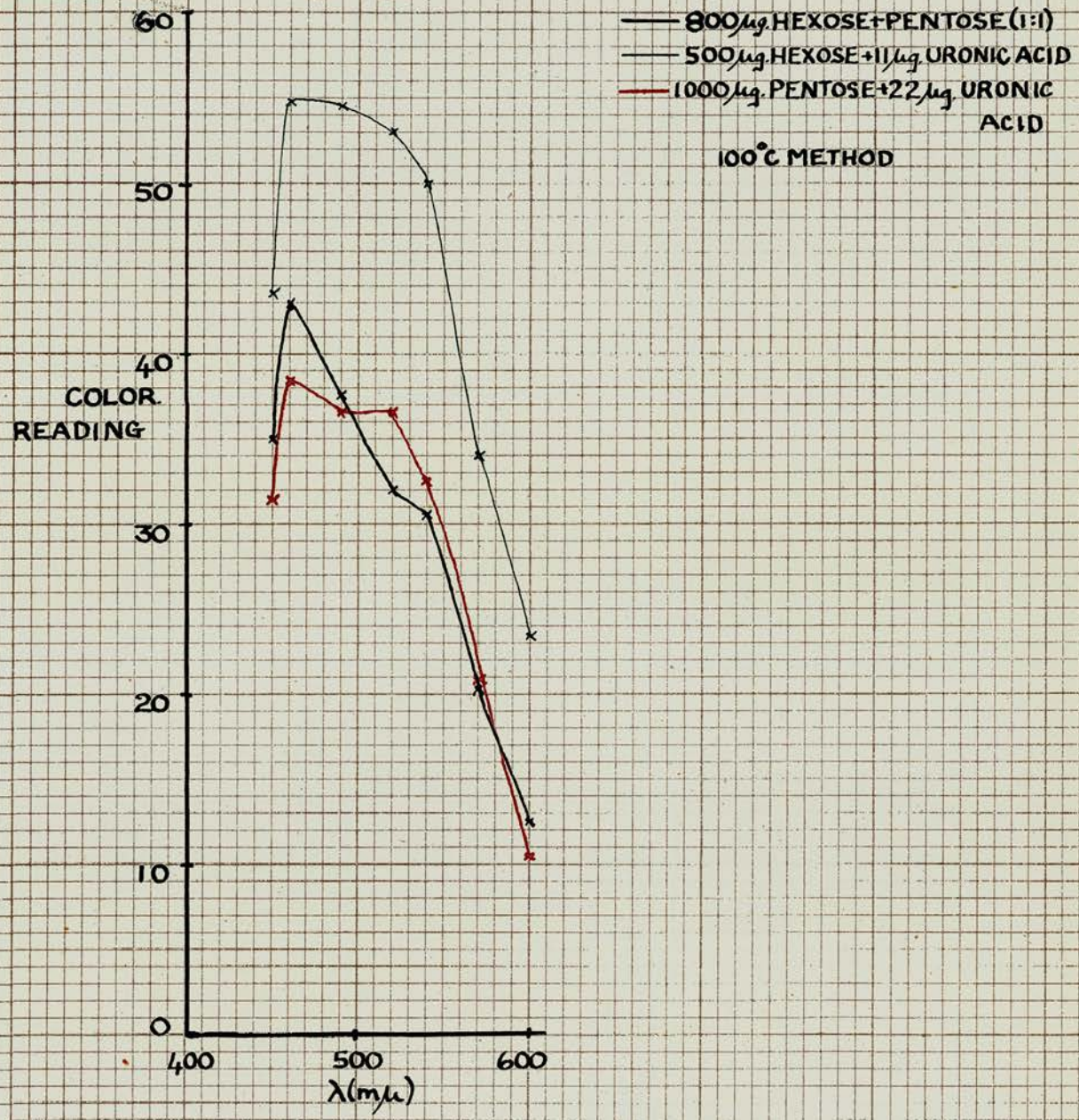


Figure VII. Absorption curves for known sugar, and sugar/uronic acid, mixtures; after prior acid treatment.

by means of the appropriate standard curves of Figs. IV & V.

Some indication as to the presence of uronic acids may be obtained through plotting the absorption curves. A few of these, constructed for various mixtures, are presented in Figs. VI & VII.

Uronic acid recovery from polysaccharide hydrolysates:- 500µg. samples of polysaccharide preparations whose relative sugar unit constitutions were known, were heated at 100°C. for 3 hr. in glass-stoppered tubes with N H_2SO_4 and known weights of galacturonic acid. After cooling, the contents of the tubes were made up to 10ml. with distilled water, and 1ml. portions taken for analysis. Recovery figures are shown in Table XIV, these having been calculated as in Table XIII except that allowance has been made for hydrolysis of the polysaccharides to free sugars.

60°C. method:- 0·4ml. portions of solution are measured into Pyrex glass tubes immersed in ice-water, 5·4ml. of a sulphuric acid solution (H_2SO_4:H_2O,6:1 by volume) are added with thorough mixing. After placing in tap water for 3 min. the tubes are immediately transferred to a water bath

TABLE XIV

Recovery of Uronic Acids from Polysaccharide Hydrolysates

Polysaccharide fraction	χ Wt. of polysaccharide (μg.)	χ Wt. of uronic acid (μg.)	Color. rdg. (total)	Color. rdg. (sugars)	Color. rdg. (uronic acid)	# Uronic acid found (μg.)	% Recovery
Oat gum (20)*	500	17	60.8	46.0	14.8	18	106
Rye gum (40)*	500	17	18.2	4.5	13.7	17	100
Wheat hemicellulose C(30) #	500	17	26.4	12.0	14.4	18	106

* See Section I

See Section IV.

χ Refers to wt./ml. of solution taken for analysis.

\# To nearest unit.

at 60°C. for 90sec. After cooling in tap water, 0.2ml. of 0.1% carbazole (ethanolic) is added with thorough mixing, whereupon, in the presence of uronic acids, a pink coloration is produced, the intensity of which increases gradually with time. Colorimetric readings are made at 520mμ against suitable blanks after 1hr. from the time of mixing with carbazole. Fig. V shows the standardisation curve for galacturonic acid and absorption curves for galacturonic and glucuronic acids. Absorption curves for sugars, with and without uronic acids, are shown in Fig. VI.

In this 60°C. procedure galacturonic acid yields a colour intensity some thirty times greater than that given by an equivalent weight of glucuronic acid (30), a finding supported by the present work. Comparison of the colour intensity produced by an unknown uronic acid can be made with a suitable standard galacturonic acid solution. Knowing the total uronide content of the polysaccharide preparation under investigation (determined by decarboxylation, or better, by the 100°C. method) comparison of the intensity produced (60°C.) with that given by an equivalent weight of galacturonic

acid will serve as a distinction between the two uronic acids. Quantitative distinction between the two acids has not been possible in the present case due to lack of correlation of sugar concentration with optical density. However, the method has been of some use in qualitative distinction, where sufficient uronide is concerned.

Estimation of uronide in the water-soluble gums:- Accurately weighed amounts of the polysaccharides (normally 5-10mg.) contained in glass-stoppered tubes (7½x1¼cm.) were hydrolysed with ca. 4ml. 2N H_2SO_4 for 3hr. periods at 100°C. After cooling to room temperature the contents were made up to 5 or 10ml. with distilled water, 0·4ml. portions being taken for the 60°C. method and 0·2-1·0ml. for the 100°C. reaction, the volume depending on constitution. Only small amounts were taken where hexose content was high, water being added to ensure a starting volume of 1ml. Blanks containing 2N H_2SO_4 in place of the hydrolysis mixtures were run simultaneously. Readings were made at 520mμ after which, knowing the relative amounts of sugar units in each polysaccharide fraction (Section I), and employing standard curves for the various sugar

TABLE XV

Uronide in the Water-Soluble Gum Fractions

Fraction *	µg. Taken for analysis	Color. rdg. (total)	Color. rdg. (sugars) χ	Color. rdg. (uronic acid)	µg. Uronic acid **	%Anhydro-uronic acid
Rye (30)	784	12.1	17.0	-	-	-
(40)	1008	14.8	15.5	-	-	-
(50)	936	9.6	14.0	-	-	-
(60)	904	13.9	13.0	-	-	-
(mother liquor)	1016	59.4	51.0	8.4	11	1
Wheat (30)	424	9.3	8.2	-	-	-
(40)	1056	29.8	15.0	14.8	18	2 ∠
(50)	728	24.0	17.0	7.0	10	1
(60)	552	29.0	26.0	3.0∠	-	-
(mother liquor)	572	29.9	20.5	9.4	12	2 ∠
Oat (20)	468	48.9	43.0	5.9∠	-	-
(20-30)	584	54.0	55.2	-	-	-
(30)	480	40.2	40.5	-	-	-
(40)	432	36.0	33.5	2.5∠	-	-
(saturation)	500	28.1	25.5	2.6∠	-	-
(mother liquor)	1032	73.3	48.8	24.5	30	3 ∠
Maize (mother liquor)	548	39.5	36.0	3.5∠	-	-
Barley (mother liquor)	1064	70.3	55.5	14.8	17	2 ∠
Malt (mother liquor)	984	46.4	49.4	-	-	-

* See Section I, Table IV.
χ From Fig. IV; correction is made for hydrolysis to free sugars.
∠ Values are too small to be positively certain.
∠ Identified as galacturonic acid by the 60°C. method.
** Calculated to the nearest unit.

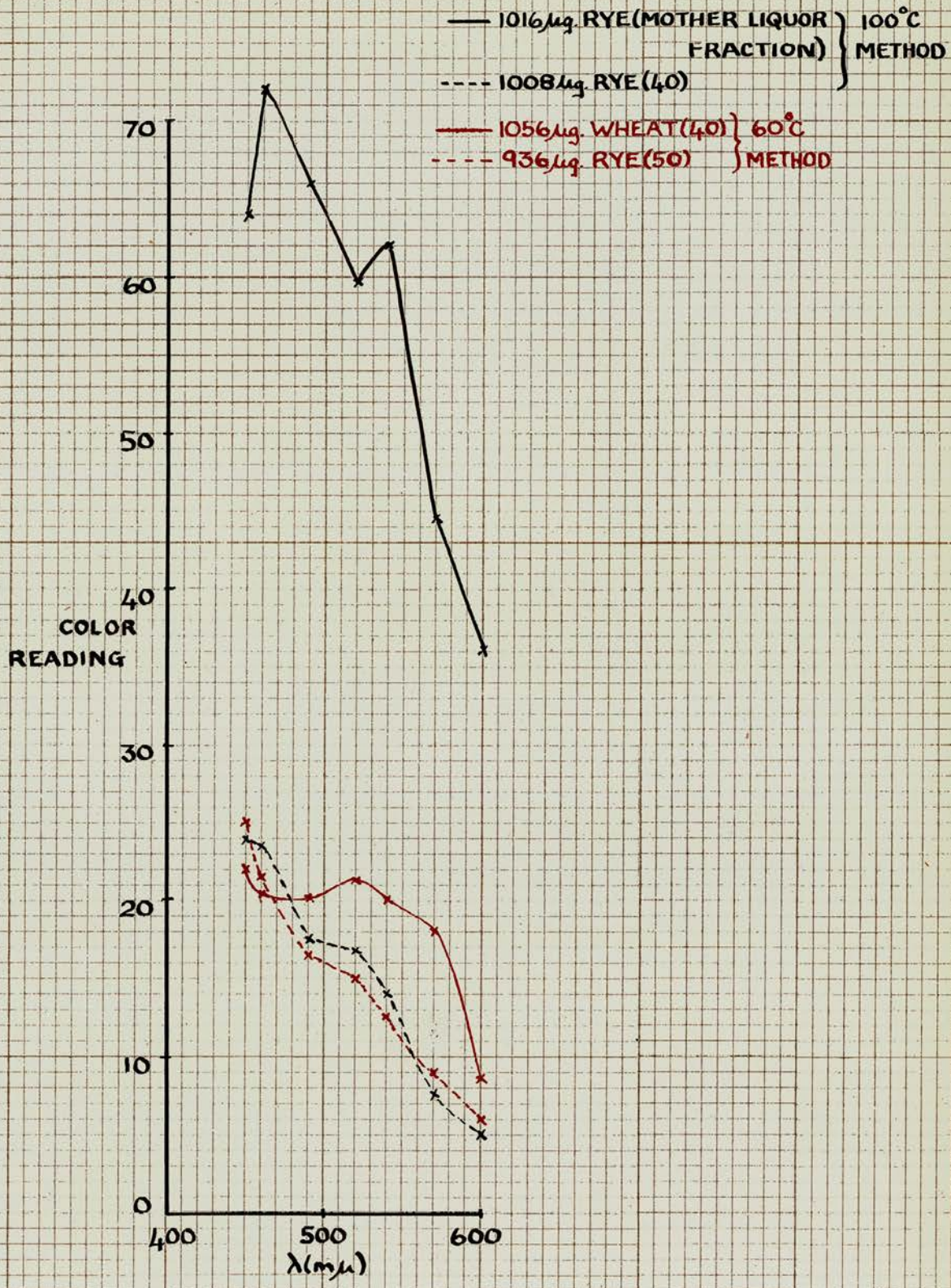


Figure VIII. Absorption curves for some cereal gum hydrolysates.

types (Fig. IV), it was possible to arrive at a value for the reading due to non-uronide carbohydrate, and thus to calculate, by difference, the amount of uronide present. Table XV contains the information obtained and some absorption curves are shown in Fig. VIII.

DISCUSSION.

Decarboxylation:- The evolution of carbon dioxide from non-uronide carbohydrate under the conditions described above has recently been reviewed by Whistler (86). However, it would seem to be a dangerous procedure to apply over-rigid corrections for extraneous carbon dioxide in view of the zero value obtained for a β-glucosan preparation (Table XII). Uronide contents of the mother liquor fractions examined show somewhat anomalous behaviour when compared with the results obtained colorimetrically. Thus, while much the same results have been obtained for the wheat mother liquor fraction, the corresponding oat fraction yields somewhat divergent values. Anomalous behaviour has also been observed in certain of the hemicelluloses described in Section IV (Tables XVI & XVII). For three such preparations, which yielded

no uronide colorimetrically, a constant value of 1.3% anhydrouronic acid was obtained by decarboxylation. While this latter technique offers a good, if somewhat tedious, means of determination, where relatively high proportions of uronic acids or uronide-containing materials are concerned, serious difficulties may arise at the lower end of the scale.

Colorimetric methods:- By means of the 100°C. procedure, uronic acids, when present in small amount (ca.2%) in admixture with true sugars, can be recovered with an efficiency of 80-100%. Even smaller proportions are determinable under favourable conditions, e.g. where interference due to sugars is small, as in the absence of hexose. Where hexose interference is too great, recoveries are not at all reliable.

Absorption due to true sugars at 520mμ is derived from two sources, namely, the reaction with H_2SO_4, and secondly, that with carbazole. As has already been pointed out, this intensity varies considerably more than is the case with hexuronic acids from one determination to another, thus making it advisable to carry out several parallel readings on the same test solution. According to Dische (29)

the absorption due to glucose (100°C. method) is proportional to concentration over the range 200-500 µg. In the present investigation the corresponding range is 200-700 µg., although it is not advisable to work at the upper end of this scale when uronic acids are to be estimated. Similar proportionality can be seen to exist for pentoses, the range being 400-1000µg.; there is also fairly good linearity between optical density and sugar concentration for hexose/pentose mixtures, experimentally obtained readings showing agreement with values calculated from the curves of individual sugars. Rather better agreement appears to exist for hexose:pentose 1:1(200-1000 µg.) than for hexose:pentose 1:2 (300-1000 µg.).

The brown or red-brown coloration which results on treating glucose under the conditions of the 100°C. method is due to the formation of humic substances of uncertain constitution, and which, according to Love (42), mask a pink coloration (λmax. 520mµ) due to complex formation involving 5-hydroxymethylfurfural. The measurement of this latter coloration has recently been employed by Mendel et al. (46) for the estimation of glucose.

Love points out the necessity for a short time of heating (less than 6 min.) in the production of the colour, a longer period resulting in masking, as above. Mendel et al. indicate the acid concentration and brand to be the primary factors (within limits), albeit with much smaller amounts (ca.150µg glucose). In the work described here, employing one batch of acid throughout, and the conditions of the 100°C. technique, no absorption maximum at 520mµ has been observed over a concentration range of 100-800 µg. of glucose. Coloration with 100 µg. or even 300 µg. is a yellowish-pink which, however, exhibits an absorption maximum at 460mµ. Moreover, heating for 5, 10, and 15 min. has not been found to cause any change in the position. It has, however, been noted that different batches of acid give definite maxima at 520mµ, even after 20min.heating, the coloration in such cases being the bluish-pink referred to by Mendel et al.(46). Such a state of affairs is suggestive of the critical nature of the H_2SO_4 employed.

Fig. IIIA shows a decreased absorption at 460mµ, for glucose, on standing for 2hr. after 20 min. heating. No such difference is apparent for

a hexose/pentose mixture, suggesting the glucose concentration to be responsible. It is evident from Fig. IIIB that the effect of carbazole on the glucose/pentose, and glucose, curves, is small but positive. On standing for 2hr. glucose exhibits a maximum from 460-490mμ while the mixture shows a similar, but larger, spread, up to about 520mμ. In the presence of galacturonic acid (Fig. IIIC) the glucose exhibits a very similar maximum and even in the presence of 5% uronic acid little information can be gained from its form. The pentose maximum at about 430mμ (not shown) may not itself be affected, but in the presence of galacturonic acid (Fig.IIID) two more maxima at 460 and 520 mμ, the latter due to the uronic acid, are produced. Unless true sugar interference is small, absorption curves determined for only a few wavelengths should not be treated too seriously.

A few possible sources of error in the application of this colorimetric technique to the polysaccharide problem are worthy of note. In the first instance figures employed for polysaccharide compositions (in terms of hexosan and pentosan) are merely relative amounts of sugar units, and

consequently, where uronide is present, readings for true sugars will be high. However, in consideration of the small uronide contents involved, and also of the relative equivalent optical densities for true sugars and uronic acids, such an error can only be very small. Secondly, and this would appear to be a more important point, it is not at all certain that readings for hexose and pentose sugars are additive outwith the ratios 1:1 and 1:2. This is especially so where less than 200µg. of glucose are concerned. Nevertheless, the high recoveries from polysaccharide hydrolysates are encouraging.

It may be claimed that the values given in Table XV represent fairly good estimates of uronide contents of the cereal gums. A few very small readings for apparent uronic acid contents are shown in Table XV and it is not certain whether they are significant. The use of the 60°C. method has served to indicate the occurrence of galacturonic acid residues in a few cases (Table XV) but the presence of glucuronide has not been shown.

This adaptation of the Dische method is scarcely suitable for general use, at any rate in its present form. It appeared the best available

means for the elucidation of the present problem and thus far it may be claimed to have proved successful. However, unless strictly interpreted, and unless standard curves have previously been constructed by the investigator, employing one suitable batch of H_2SO_4 throughout, the results might easily lead one to highly erroneous conclusions. A large measure of experience is essential before analyses can be successfully embarked upon. At present it is felt that the use of an EEL colorimeter is justified since a more sensitive instrument might serve to magnify the variations in certain of the readings as mentioned above. Even so, it might be of advantage to follow up this work with a more accurate study of absorption curves both in the visible and ultra violet regions of the spectrum.

Uronide in the cereal gums:- The apparent participation of uronide in at least four of the mother liquor fractions is noteworthy, especially since, in three of these cases, galacturonic acid is indicated. This is not inconsistent with the overall constitution of these fractions suggesting as they do, pectic substances. Whether or not polygalact-

uronic acid will prove to be present remains to be seen. In this connection it is of interest to note that, in the maize and barley malt fractions, containing little or no galactan, the presence of uronide is very doubtful. Such an observation may be in line with the galactan-uronide relationship seen to hold on electrophoresis (Section II).

Besides these highly soluble fractions the only others in which uronic acid (galacturonic) is definitely present are those precipitable at 40 & 50% salt from wheat. This immediately recalls Perlin's finding of uronide in his wheat gum preparations (64).

In view of the foregoing results, and if the mother liquor fractions be dismissed as not representing 'true' cereal gums, only very minute amounts of uronide can occur in the latter substances. Such a finding adequately accounts for the virtual absence of uronic acids in gum hydrolysates rather than any loss in other directions.

FRACTIONATION OF SOME CEREAL HEMICELLULOSES

INTRODUCTION

In view of the likely structural relationship between the water-soluble gums and the initially water-soluble hemicelluloses (see General Introduction) it was deemed desirable to investigate the properties of the latter after extraction from the grain and to compare them with those of the gums. Such an investigation should be especially interesting since information has become available on the production of considerably increased gum yields from tissues pretreated with alkali (71) or with enzymatically-active cereal extracts (71, 72, 74). The work described below is solely concerned with those hemicelluloses which lend themselves to easy extraction from the grain, a necessary point to bear in mind throughout. The opportunity has been taken of applying the ammonium sulphate fractionation technique to the present problem and by such means it was hoped to bring about a fairly direct comparison between the hemicelluloses and gums. In this way certain information has been sought in an effort to justify further the idea that any distinction between the

hemicelluloses and gums is chemically artificial, the only essential difference being the possession or otherwise of initial water solubility.

EXPERIMENTAL

Materials:- Raw materials were employed as follows: whole barley grain, barley husk (approx. 7% of the grain) substantially endosperm-free and prepared by the method described by Mackenzie (73), and pearl barley (approx. 44% of the whole grain and representing substantially pure endosperm); whole wheat and wheat flour ("C flour", said to represent the purest endosperm material commercially available); oat husk.

Preparation of hemicellulose extracts:- 100 g. portions of the ground materials were added with stirring to 400 ml. volumes of boiling water until a fairly smooth paste was obtained. Homogenization was completed by subjecting the mixture to autoclave treatment (10 lb./sq.in.; 15 min.). Removal of starch, a necessary step at this stage, was carried out by treatment with α-amylase at 65°C. For this purpose 100 g. malt were extracted with 500 ml. water at room temperature for 3 hr. with stirring. After centrifuging and filtering, 0.2 g. calcium

acetate were added per 100 ml. filtrate, the latter being maintained at 70°C. for 15 min. and then cooled to 65°C. Such treatment results in the substantially, if not wholly, complete inactivation of the enzymes present in the malt extract, with the exception of ∝-amylase. The prepared malt extract was then incubated with the grain homogenate (brought to 65°C. before addition) at 65°C. for 2 - 3 hr. After this initial treatment the grain residue was filtered off through muslin, resuspended in water as described above, and treated with a fresh ∝-amylase extract in a similar fashion. This second incubation normally occupied 3 - 5 hr. in the case of whole barley, whole wheat, pearl barley and wheat flour, husk material requiring a less lenghy treatment. When a drop of the cooled reaction mixture failed to yield any coloration with an iodine solution the final grain residue was filtered off, washed thoroughly with boiling water (ca. 5 litres per 100 g. original grain) to remove soluble dextrins etc., and dried in air. The dried material was then extracted with successive 500, 250 and 250 ml. of 4% NaOH per original 100 g. grain for 20 min. in each case at room temperature with

constant stirring. The mixtures were centrifuged, the brown centrifugates combined, and filtered twice rapidly through paper pulp to give a clear solution.

Fractionation:-All steps were carried out as rapidly and at as low a temperature as possible. Filtered extracts were immediately subjected to fractionation. Initially, fractionation with acid (HCl) and acetone was attempted by the method of Norris & Preece (54), acidification etc. being performed in a water-ice mixture. Fraction A was obtained from wheat flour only, C being recoverable also from this source; preparations from wheat flour proved very troublesome owing to large losses of material during filtration. Fractions B and C were both obtained from each of the two cereals barley and wheat; in the remaining cases, fraction B was absent, or so small as to be unworkable, and was recovered along with C. The products were white or cream-coloured substances, those from wheat and barley endosperm being exceedingly fibrous. All were water-soluble to a greater or lesser extent but it was noticeable in certain cases that separation occurred even from

fairly dilute aqueous solution on short standing. Ash and moisture contents, where determined, were of the same order as for the water soluble gums. Yields and physical properties are shown in Table XVI. Relative amounts of anhydro-sugar units (determined as in Section 1) are also given in this Table together with uronide contents determined as in Section III (see also Fig. IX). Hydrolysis with N H_2SO_4 gave no visible products other than glucose, arabinose, and xylose.

Ammonium sulphate fractionation (42) was applied to certain C fractions as shown in Table XVII. In general, workable amounts were obtained at 30, 40 and 70% salt concentrations (the latter representing saturation). Very small precipitates obtained at 50 and 60% were isolated together with the saturation level fractions. Whole barley yielded a fraction at 20%, one precipitating between 20 and 30%, and a further fraction from 30 - 40%. In general, and apart from the latter two fractions, precipitation steadied up after 3 - 4 salt treatments. Mother liquor fractions in small amount were obtained from all sources.

Compositions were determined by the usual

TABLE XVI

Yields, Characters, and Compositions of some Cereal Grain Hemicellulose Fractions.

Source Material	Fraction *	Yield (% of source material	Specific Viscosity (25°C.)	$[\alpha]_D$ (°) (15°C., 0.5%)	Anhydro-Sugar Units (%)			Anhydro-Uronic ≠ Acid (%)
					Gluco-san	Arab-an	Xy-lan	
Whole Barley	B	0.07	8.2	-48 χ	37	22	38	2
	C	2.05	6.5	-80 χ	34	22	44	0 (1.3)
Barley Husk	C	3.60	2.0	-82	6	15	77	2 (1.8)**
Pearl Barley	C	3.12	16.1	-4	77	6	17	0
Whole Wheat	B	0.18	3.7	+	26	6	68	0
	C	1.67	11.4	-80	18	21	60	0
Wheat Flour	A	0.46≠	+	-52	73	0	27	0
	C	0.24≠	11.0	-80	19	31	50	0
Oat Husk	C	1.42	0.5	-76	5	22	69	4 **

* Norris & Preece fractionation (11)
≠ Considerable losses inevitable in preparation.
\+ Not determined.
χ In aqueous soln., others in 4% NaOH.
≠ Values in brackets determined by decarboxylation; others determined by the colorimetric method of Section III.
** Galacturonic acid definitely indicated by 60°C. method. Some absorption curves of interest are given in Fig. X.

TABLE XVII

Characters and Compositions of some Hemicellulose Subfractions.

Source material and fraction *	Subfraction pptd. by $(NH_4)_2SO_4$ in % concn. shown or by acetone	Yield (% of source material)	Specific Viscosity (25°C.) 15°C. 0.5% soln.		Anhydro-Sugar Units (%)			Anhydro-Uronic Acid (%) * ≠
					Glucosan	Arabinan	Xylan	
Whole Barley C	20	0.066	3.0	+	90	0	10	+
	20-30	0.215	2.1	-44	72	8	20	+
	30	0.201	1.9	-52	58	12	30	0
	30-40	0.443	1.8	-82	33	21	46	0
	70	0.191	1.1	-116	5	28	66	ca.1
	acetone	0.066	0.1	-58	8	27	64	ca.1
Pearl Barley C	30	0.089	+	+	95	0	5	0(1.3)
	40	1.210	4.7	-124≠	93	0	7	0
	70	0.278	2.9	-104≠	21	20	59	0
	acetone	0.214	0.4	+	36	22	42	0
Whole Wheat C	30	0.469	3.8	-68	27	18	55	0(1.3)
	40	0.037	4.1	-132	4	32	64	+
	70	0.075	3.2	-108	0	42	58	ca.1
	acetone	0.063	0.1	+	0	37	62	ca.1
Oat Husk	30	0.336	0.5	-132	15	15	68	1**
	40	0.118	0.5	-120	6χ	15	76	2**
	70	0.053	+	+	4	18	72	6
	acetone	0.107	0.2	+	7	18	72	3

* See Table XV.
≠ In 2% NaOH, others in aqueous soln.
\+ Not determined.
χ Part at least consists of galactose.
** Galacturonic acid definitely indicated by 60°C. method.
≠ Values in brackets determined by decarboxylation.

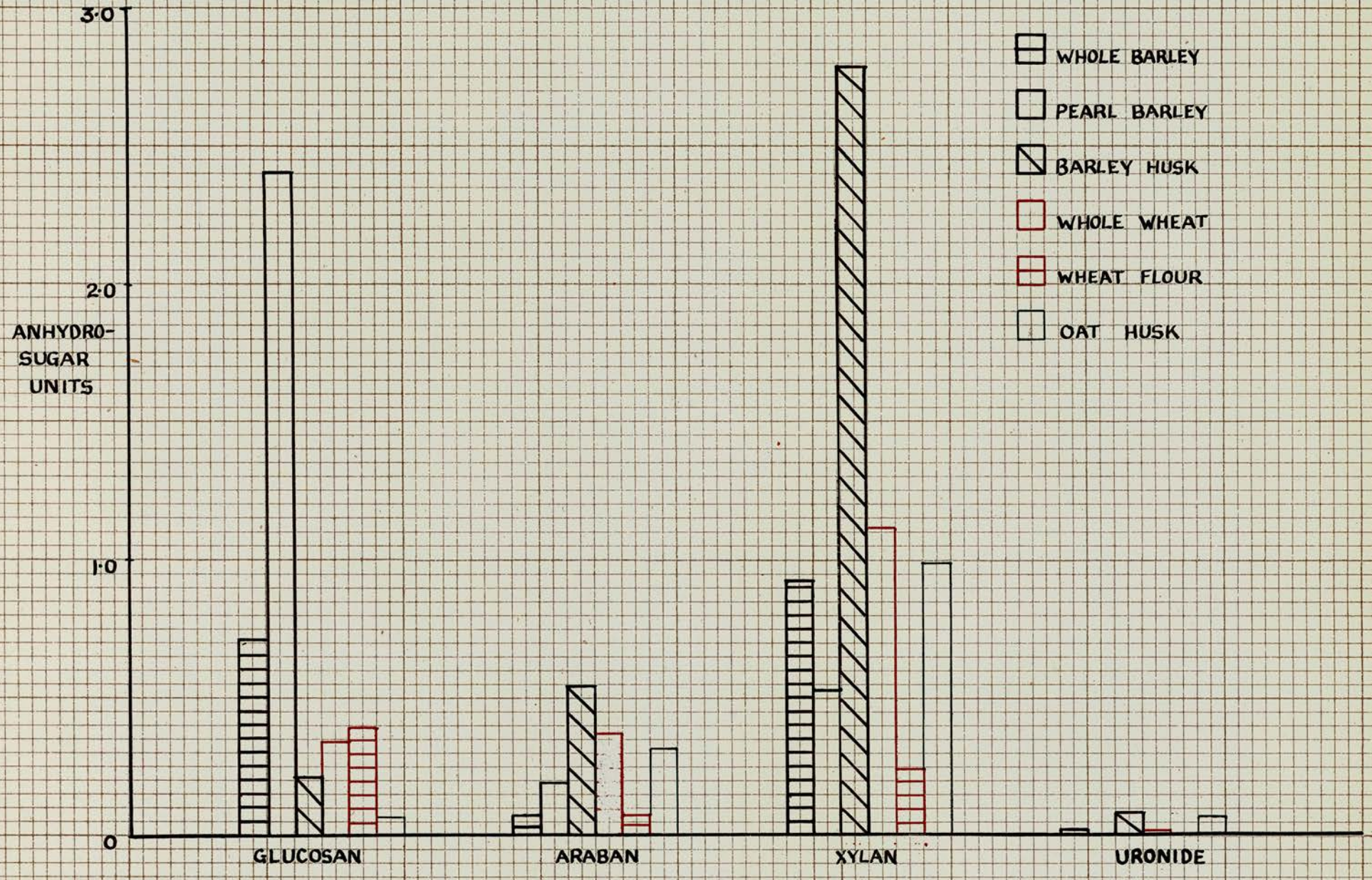


Figure IX. Hemicellulose sugar units as % of dry source materials; before fractionation.

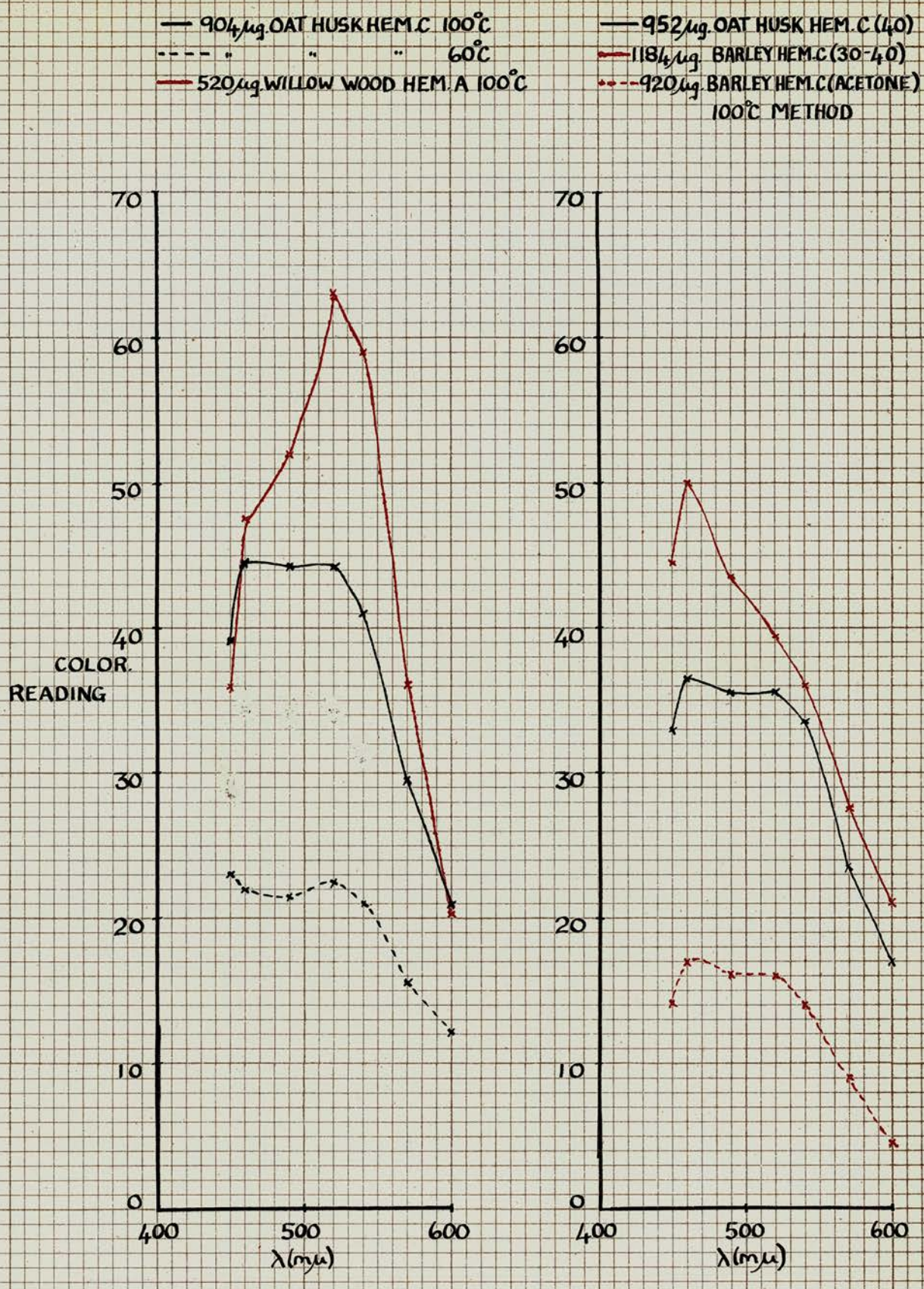


Figure X. Absorption curves for some hemicellulose hydrolysates.

methods and are shown, together with yields and some physical properties, in Table XVII. N H_2SO_4 hydrolysis for 3 hr. gave, with one exception, one or more of the three sugars, glucose, arabinose, and xylose. The exceptional case was that of the oat husk fraction obtained at saturation level which gave a spot having R_F = 0.14 - evidently representing a uronic acid, and another R_F = 0.16 apparently representing galactose. The chromatogram of this hydrolysate is shown in Plate II, but unfortunately the galactose and/or glucose spot is only just visible while the uronic spot is not detectable. Also, two products of partial hydrolysis are just visible, these possibly containing uronide material. Fig. X contains some absorption curves for these hemicellulose subfractions. The curve for a hemi-cellulose preparation (ca. 13% uronide) from willow wood is given for comparison (for preparation see Section V.). In Table XVIII are shown the xylan:araban ratios for the principal pentosan-rich hemicellulose fractions and subfractions.

Fractionation losses:- The usual losses were obtained during salt fractionation, some 40 - 60% of

TABLE XVIII.

Ratios of Xylan to Araban in some Hemicellulose Fractions (Figures shown are calculated from the results of Tables XV and XVI).

Source	Fraction	Xylan:Araban
Whole Barley	B	1.70
	C	2.00
	C (20 - 30)	2.40
	(30)	2.40
	(30 - 40)	2.20
	(70)	2.40
	(acetone)	2.40
Barley Husk	C	5.25
Pearl Barley	C	2.85
	C (70)	3.00
	(acetone)	2.13
Whole Wheat	B	11.50
	C	2.80
	C (30)	3.00
	(40)	2.00
	(70)	1.38
	(acetone)	1.68
Wheat Flour	C	1.63
Oat Husk	C	3.17
	C(30)	4.56
	(40)	5.31
	(70)	4.00
	(acetone)	4.00

TABLE XIX

Hemicellulose Loss during Salt Fractionation.

(Results expressed in anhydro-sugar units as % of source material).

Source	Fraction	Glucosan	Araban	Xylan
Whole Barley	hemicellulose C*	0.697	0.451	0.902
	" "χ	0.488	0.205	0.482
Whole Wheat	" "*	0.301	0.351	1.002
	" "χ	0.128	0.151	0.365
Pearl Barley	" "*	2.402	0.187	0.530
	" "χ	1.345	0.053	0.343
Oat Husk	" "*	0.071	0.312	0.980
	" "χ	0.066	0.097	0.412

* Refers to unfractionated preparations.

χ After fractionation; refers to total for fractions in each case.

the initial crude mixtures being recovered in each case. Great drops in the viscosities of the highly viscous preparations were also found to occur (see Tables XVI and XVII). Table XIX contains data on the effect of salt fractionation on the recoveries of the individual sugar unit types. ~~and uronide.~~

Partial acid hydrolysis of some hemicellulose fractions:- The procedure was exactly that employed in the case of the water-soluble gums (Section I). The results obtained are set out in Tables XX and XXI, while Plate IV shows some of the chromatograms obtained.

Three of the pentose oligosaccharides detected in the above work were subjected to further investigation. These had R_F values of 0.08 (BuOH-Ac-H_2O), and 0.004 (BuOH-EtOH-H_2O); 0.12 (BuOH-Ac-H_2O) and 0.035 (BuOH-EtOH-H_2O); 0.070 (BuOH-EtOH-H_2O). Sufficient amounts of the first two were recoverable from paper chromatograms for the purpose of iodine titration before and after hydrolysis and also for the identification of hydrolytic products. In this way these two substances were found to correspond to certain of those appearing on chromatograms of partially hydrolysed

Table XX.

Partial Hydrolysis of some Hemicellulose Fractions-
(H denotes hexosan; P denotes pentosan material)

Fraction	Normality of H_2SO_4 *	Butanol:acetic acid:water		Butanol:ethanol:water	
		Carbohydrates on chromatograms	R_F value	Carbohydrates on chromatograms	R_F value
Barley C(30) Wheat C(70)	0.1, 0.2	P P P arabinose✓ + xylose	0 (diffuse) 0.12	P ✓ P arabinose✓ xylose	0 0.035
Wheat C	0.02			P P P P ✓ arabinose xylose	0 0.005 0.035 0.070
Barley Husk C Oat Husk C	0.1,0.2	P P P P arabinose xylose	0 (diffuse) 0.08 0.12	P P H P arabinose xylose	0 0.004 0.020 0.040
Barley Husk C	0.02			P P P P arabinose xylose	0 0.005 0.035 0.064

* Normality employed for hydrolysis of 2 ml. gum solution, temp. of hydrolysis = 100°C.
✓ Glucose present in hydrolysate of barley C(30).
\+ Trace of pentose oligosaccharide R_F 0.17 in hydrolysate of wheat (70); 0.1N acid.
✓ Trace present.

Table XXI.

Partial Hydrolysis of some Hemicellulose Fractions

(H = hexosan; P = pentosan)

Fraction	Normality of H_2SO_4	Butanol:acetic acid:water		Butanol:ethanol:water	
		Carbohydrates on chromatograms	R_F value	Carbohydrates on chromatograms	R_F value
Pearl Barley (30-40)	0.1, 0.2	H*	0	H*	0
		P	0.12	P	0.004
		glucose		H	0.020
		arabinose		P	0.035
		xylose		glucose	
				arabinose	
				xylose	
Wheat flourC	0.1, 0.2	P	0	P	0
		P	(diffuse)	P	0.004
		P	0.12	P	0.035
		P	0.14/	glucose*	
		glucose*		arabinose	
		arabinose		xylose	
		xylose			
	0.02			P	0
				P	0.005
				P	0.035
				P*	0.070
				arabinose	
				xylose	

* Trace present.

/ 0.1 N acid.

gums (Section I). That having the lower R_F values was identified as a xylotriose and the other as a xylobiose. Insufficient of the third substance was recoverable for iodine titration but on hydrolysis both arabinose and xylose appeared. From its position on chromatograms a disaccharide would appear to be indicated.

DISCUSSION

It is felt that it cannot be over-emphasised that the hemicelluloses investigated above do not by any means repfesent the total occurring in the grains but merely those portions which are relatively easily extracted. Whole barley contains some 8% pentosan of which the soluble gum and the hemicelluloses now extracted account for only about one-fifth. Thus the more resistant materials, presumably existing in combination with lignin or other substances in the cell wall, are excluded from the present investigation.

It seems from the figures given in the above Tables that the husk hemicelluloses differ markedly from those of purely endospermic origin. Thus, in the case of unfractionated husk preparations, large xylan:araban ratios are evident and

little glucosan is present. Also, these are the only fractions possessing relatively large amounts of uronide. In pearl barley and wheat flour preparations on the other hand, uronide is scarcely detectable, xylan:araban ratios are significantly lower, and substantial proportions of glucosan are present. Whole grains tend to give values of an intermediate nature. In general it would appear that pentosan hemicelluloses, like uronide material, are relatively more abundant in the husk and bran than in the inner endosperm.

As has already been mentioned (Section I) it is inadvisable, particularly at present, to attribute over-much importance to viscosity measurements. However, it is believed legitimate to make some broad comparison of the various preparations since identical preparative methods were applied in each case. With the above caution in mind it is at once obvious that the two husk hemicelluloses isolated have viscosities very much less than those isolated from the two endosperms. There appears to be no immediate reason for this large (approx. eightfold) difference. Fraction C from

wheat flour is strikingly similar in character and composition to fraction C from whole wheat but there are obvious differences between fractions C from whole and pearl barley. Such variations in the latter case must be due in some measure to the presence of husk-type hemicelluloses in the whole barley preparation. Such an idea presumes the existence of two hemicellulose types and while this is the most probable conclusion which can be drawn from the results obtained it is well to bear in mind that only two husk and two endosperm preparations have been considered. Even so this presents a useful working hypothesis which may form the basis for future work on the subject. Assuming the husk type to have structural significance it would be reasonable to suppose it to be dispersed throughout the grain, being particularly abundant in the husk and tissues outwith the aleurone layer; nevertheless, complete absence from the inner endosperm would be unlikely. The endospermic type on the other hand would be particularly abundant in the inner endosperm and substantially absent from the husk. Such a view is not inconsistent with the experimental results presented.

Fractionation:- A fair measure of success has been achieved by the application of the salt fractionation technique. In the four cases investigated 73 - 78% of the total hemicellulose recovered by salt fractionation is precipitated at a salt concentration up to and including 40%. Experience with the method suggests that fractionation of these hemicelluloses is not so wide as that obtained for certain of the water-soluble gums.

In no case has a fraction of such purity as the water-soluble β-glucosan been obtained though in some cases this ideal has been closely approached. On the other hand two fractions apparently devoid of glucosan have been obtained from wheat and a number of fractions with minimal glucosan content from oat husk. It is consistently true, with the exception of some mother liquor fractions, that glucosan content of the fractions diminishes as salt concentration increases; simultaneously, araban content tends to increase. However, for the whole barley fractions the xylan: araban ratio remains substantially constant at a value of 2.2 - 2.4. This observation is again not in agreement with Perlin's view for the wheat

pentosan (64), and, while the latter's theory seems to be worthy of acceptance on certain grounds there must be other factors, such as a decrease in chain length, which could also contribute towards increased solubility.

As with the gums large losses are evident on salt fractionation, viscosity decrease being an accompanying feature. At present it would appear that the losses are largely mechanical. However, for convenience, this aspect of the problem will be deferred until the following Section. Certain unexplained changes in specific rotation are also apparent on salt treatment. Thus crude hemicellulose preparations normally possessing specific rotations of -50° to -80° give rise on fractionation to certain fractions possessing rotations of the order found for the rye pentosan. However, that such phenomena are not entirely confined to the hemicelluloses is indicated by the unfractionated gums of Preece & Mackenzie (73). These have fairly low negative rotations whereas the fractionated gums described in Section I possess very much more highly negative values, especially where the pentosan-rich fractions are concerned. It would appear

then that the gums and hemicelluloses of the type discussed herein are affected in a similar manner by the salt fractionation procedure.

Hemicellulose-gum relationships:- Of the cereal gums examined only those from the mother liquor and a few from wheat have been shown with certainty to contain uronide material. Thus the 'true' water-soluble gums resemble the present endosperm-type hemicelluloses in the virtual absence of uronide. Furthermore the richness of the barley gums in glucosan is paralleled by a similar richness of the barley endosperm hemicelluloses. Investigations (5) have shown the presence of 1, 3 and 1, 4 β-linkages in the water-soluble barley glucosan while in the present work partial hydrolysis has enabled the detection of substances with similar chromatographic properties to cellobiose and laminaribiose from the same source. Also, a substance correponding to cellobiose (R_F 0.02, BuOH-Ac-H_2O) has been observed in hydrolysates of the barley endosperm glucosan (Table XX) but any laminaribiose would be completely masked by the large amount of xylobiose present. The presence of these hexose oligosaccharides from gums and

hemicelluloses suggests a structural resemblance between the two classes, as to a certain extent does the difficulty in hydrolysis of the glucosan in comparison with the pentosan material.

The hemicelluloses of wheat endosperm are richer in pentosan material than are those from the corresponding barley tissue. Nevertheless, one fraction (A), which consists of almost 75% glucosan has been isolated from wheat flour. The experimentally observed rotation (-52°) suggests the presence of approximately three parts of β-glucosan, $[\alpha]_D^{15}$ = ca. -12° and one part of pentosan $[\alpha]_D^{15}$ = ca. -135° (calculated value = -45°). While wheat gum itself is substantially (probably completely) free from β-glucosan the indications are (77) that autolysis of wheat leads to the production of glucosan-rich water-soluble material, at least part of which is laevorotatory. Such information, although of a preliminary nature, seems to furnish additional evidence in favour of a relationship between the water-soluble and insoluble β-glucosan material.

For discussion purposes it will be tentatively assumed that the glucosan present in pentosan-rich hemicellulose fractions exists merely

as a concomitant and plays no part in a mixed molecular species. Partial acid hydrolysis has thrown some light on similarities existing between the pentosan gums and hemicelluloses as well as the hexosan polysaccharides. As already mentioned the relative ease of hydrolysis of the pentosan hemicelluloses is similar to that found for the pentosan gums. Also, similar products are obtained, arabinose being preferentially removed under relatively mild conditions whereas on more vigorous treatment a series of xylose-containing oligosaccharides are produced. Xylobiose and xylotriose are known to be among these present. On hydrolysis with 0.02N acid, however, the hemicelluloses yield one oligosaccharide not produced in the case of the gums (see Plate IV) and which appears to contain both xylose and arabinose. Experience suggests that this product is mainly present in hydrolysates of husk-type hemicelluloses but is also found to a certain extent in all the others treated. What significance can be attached to this finding is not at all clear.

As already stated the hemicellulose fractions obtained from whole barley (except where

arabinose is substantially absent) possess constant xylan:araban ratios, a state of affairs not entirely inapplicable to the pearl barley fractions. Thus, there is no apparent relationship between the ratio and solubility. It would appear that hemicellulosic pentosan material is liable to precipitation at lower salt concentration than is found for the gums; moreover, the xylan:araban generally tends to be somewhat higher with the hemicelluloses than with the gums. While this latter observation may, in part at least, be due to the presence of husk hemicelluloses, it can be shown (72) that the ratio decreases among gums in passing from barley, through enzyme-modified barley, to malt. Thus the present observation in the case of the hemicelluloses might form the beginning of such a trend. Furthermore, the autolysis gums of Preece & Aitken (74) contain somewhat similar proportions (ratio ca. 1:1) of xylan and araban, in which respect they resemble the malt gums. There thus appears to be an elimination of xylose residues or a preferential production of soluble arabinose residues. There is a possibility that such an effect could be brought about by transglycosidation of arabinose

residues from one arabo-xylan molecule to another, thus decreasing the solubility of the first and increasing that of the second. Simple shortening of arabo-xylan chains cannot wholly explain the effect unless free araban is available. However, it seems very likely that decrease in chain length does occur as one of the various reactions involved.

The wheat hemicelluloses fractionated from hemicellulose C show, as did the water-soluble wheat gums, a decrease in xylan:araban ratio with increasing water solubility; both groups of wheat product (unlike both groups of barley product) accordingly conform to the Perlin principle which was initially formulated after studies on wheat polysaccharides. Again the ratios tend to be slightly higher for the hemicelluloses than for the gums; however, it must be borne in mind that whole wheat hemicellulose was fractionated, thus introducing bran constituents. On the basis of preliminary work (77) it is already known that autolysis of wheat yields progressively increasing amounts of water-soluble pentosan in which the xylan:araban ratio is similar to that in the most soluble wheat hemicellulose and gum fractions.

Furthermore, salt fractionation of one of these gum mixtures (30 min. autolysis) gives pentosan-rich fractions from the 30% to the saturation precipitation levels in which the xylan:araban ratio decreases once more (ca. 4.5:1 at 30%, 1.6:1 at saturation) with increasing solubility. This seems therefore, to be a constant effect for wheat and suggests some difference in the pentosanase potentialities of this cereal as compared with barley.

EFFECTS OF PREPARATIVE METHODS ON POLYSACCHARIDES

INTRODUCTION

The effect of alkaline conditions on polysaccharides has been mentioned frequently above. Widespread use has been made of alkalies in the preparation of these substances and although losses have been recognised little or no specific work appears to have been done on the manner in which such losses can occur. In this connection it is interesting that Angell and Norris (3) substituted the milder cupric sulphate-glycerol precipitant for Fehling's solution. The more physical method of salt fractionation (72) has also been noted to fall short where recovery is concerned. It is thus highly desirable to investigate the manner in which losses of this sort are possible.

A series of investigations as to the relative merits of various preparative methods are described below, and the opportunity has been taken of comparing the behaviour of the cereal gums and a typical wood hemicellulose under similar conditions.

EXPERIMENTAL

In the work described below whole barley

grain was employed as a source of water-soluble gum while a sample of willow wood sawdust formed the source material of a hemicellulose.

Inactivation of enzymes and preparation of extracts: Inactivation of the ground barley enzymes was carried out by treatment with boiling 80% aqueous ethanol for two 30 min. periods. After drying in air the residue was extracted for three 30 min. periods with 2½ times its weight of water at 40°C. The aqueous extracts were combined and taken to a small volume after which it was filtered bright. Willow sawdust (200g.) was extracted under reflux on a boiling water bath for four 30 min. periods, with, in all, 4½ litres of 0.5% ammonium oxalate solution to remove water-soluble materials and pectic substances. The residueswas extracted with 4% NaOH(ca.2½ litres) for three 30 min. periods at room temperature, stirring being continued throughout. The alkaline extract was finally filtered bright through paper pulp.

Fehling's precipitation:- The prepared aqueous extract, or a solution of barley gum, as the case may be, was treated with an equal volume of Fehling's solution, and acetone was added to 40%

by volume. The precipitated copper complex was centrifuged off, decomposed with 2N HCl and the free gum recovered by bringing the acid solution to 60% with respect to acetone. After washing with three 100 ml. portions of 60% acetone, and three 50 ml. portions of 95% ethanol, the product was dried off in vacuo for two or three days.

In the case of the hemicellulose, an alkaline extract or solution was treated with an equal volume of Fehling's solution, and the copper complex filtered off on muslin, or centrifuged off. The alkaline mother liquor was treated with acetone (to 40%) and any further precipitate removed in the same way. These complexes were then recovered exactly as for the gums, except that, in the case of the one recovered with Fehling's solution alone, a hemicellulose fraction (corresponding to hemicellulose A, Norris & Preece, 54) designated H_1, was insoluble in acid, while a second fraction (corresponding to hemicellulose C), termed H_2, was recoverable from the acid mother liquor.

Copper sulphate-glycerol precipitation:- Solutions of gum (aqueous) or of hemicellulose (4% NaOH) were mixed with 1/10 of their volume of M $CuSO_4$ and 1/20 of their volume of glycerol. The

copper complexes formed were decomposed with 50 ml. of 25% acetic acid in each case. The free polysaccharide was precipitated by bringing the solution to 60% with respect to acetone followed by drying off as above.

Ammonium sulphate precipitation:- Gum solutions or extracts were saturated with ammonium sulphate and the precipitated gum contrifuged or filtered off. This was then redissolved in water, dialysed, and recovered with acetone prior to drying off.

Glucose equivalent determination:- Suitable volumes of gum extracts or ca. 20 mg. portions of polysaccharide material were hydrolysed for 3 hr. with N H_2SO_4 (ca. 1mg./ml.). Cold hydrolysates were neutralized to methyl orange with N NaOH and the solutions made up to 50 ml. with distilled water. 10 ml. volumes were treated with 40ml. portions of 95% ethanol and the precipitated Na_2SO_4 removed by filtration. After concentration in vacuo the filtrate was investigated for reducing groups (expressed as glucose equivalents) by the Somogyi method (82). Where HCl was employed for hydrolysis the neutralized solution was used directly for reducing group determination.

The effect of Fehling's precipitation on cereal gum extracts: An aqueous extract of barley was prepared and its glucose equivalent determined. Part of the extract was subjected to dialysis against tap water (3 days) after which the dialysate (A_1) and non-diffusible portion (A_2) were examined for glucose equivalents. A further portion of the extract was used for a Fehling's precipitation, the glucose equivalents of the recovered gum, the alkali-acetone mother liquor (after dialysis) and the acid-acetone mother liquor being estimated. A portion of this prepared gum was dialysed in solution and the glucose equivalents of the dialysate (B_1) and the non-diffusible fraction (B_2) were again determined. Another grain extract was prepared, part of which was treated by the Fehling's method, the recovered gum (C) and the dialysed acid-acetone liquor (C) being investigated for glucose equivalents. A second part of this extract was dialysed before Fehling's precipitation, the recovered gum (D) and the acid-acetone (D) then being investigated for glucose equivalents without dialysis. Results are summarised in Table XXII and Fig. XI, all figures being presented as glucose equivalents. Each value is an average of at least

TABLE XXII

Distribution of Carbohydrate on Fehling's Precipitation

Solution or sample	Glucose equiv. as % of total carbohydrate in extract	Glucose equiv. as % of dry grain	Solution or sample	Glucose equiv. as % of total carbohydrate in extract	Glucose equiv. as % of dry grain
Grain extract	100	0.84	Grain extract	100	0.92
Solution A_1	20	0.17	Fehling's gum C	65	0.59
Solution A_2	78	0.65	Acid-acetone C	3	0.02
Fehling's gum	62	0.52	Fehling's gum D	66	0.60
Alkali-acetone	0	0.0	Acid-acetone D	5	0.04
Acid-acetone	6	0.05			
Solution B_1	0	0.0			
Solution B_2	59	0.50			

After dialysis.

TABLE XXIII.

Comparison of Salt and Fehling's Precipitation Methods

Sample	Method of recovery	% Recovery
Fehling's gum	$(NH_4)_2SO_4$ pptn.	75
$(NH_4)_2SO_4$ gum	Fehling's pptn.	92

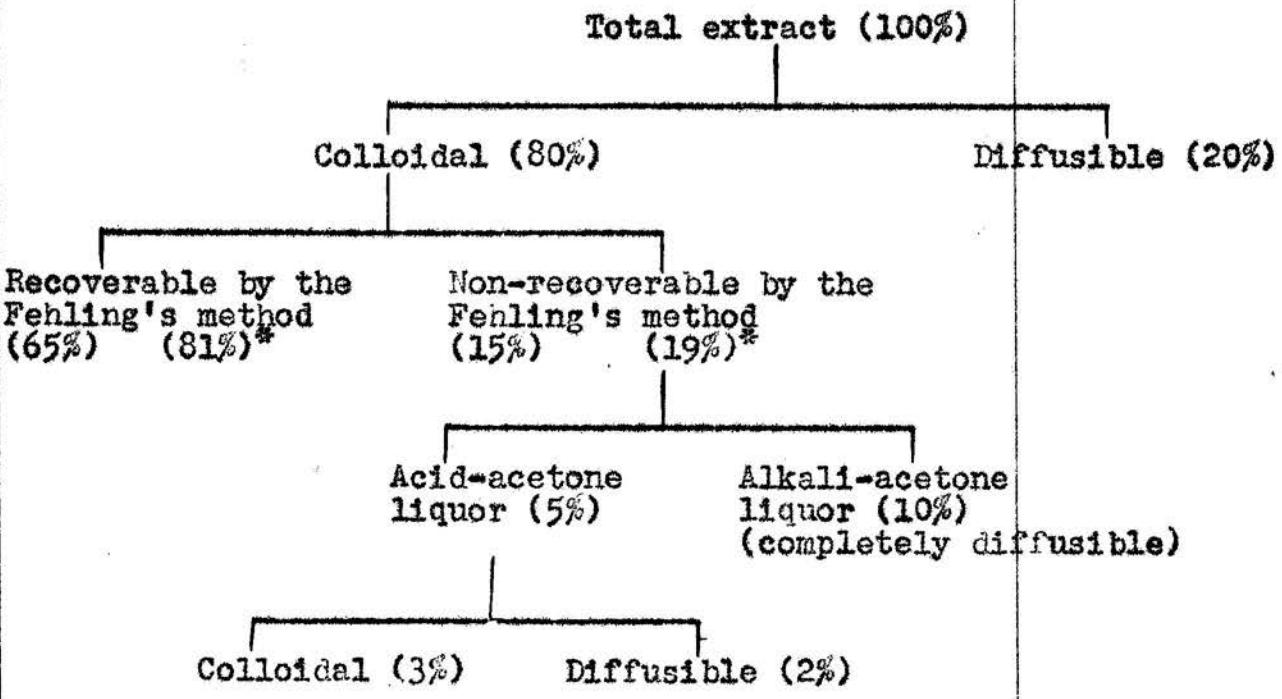


Figure XI. Distribution of carbohydrate after precipitation of an aqueous extract of barley by the Fehling's method.

* Refers to % of colloidal carbohydrate.

three determinations.

The effect of preparative methods on isolated gums:- In view of the apparent absence of colloidal polysaccharide from the alkaline mother liquor, and of the appreciable reducing power of the acid-acetone liquor, it was felt that a reprecipitation, by the Fehling's method, of a gum recovered by means of the Fehling's procedure, might prove to be of interest. This resulted in a recovery of 88%, the acid-acetone liquor exhibiting virtually no reducing power (see Fig. XII). The nature of the carbohydrate present in the acid-acetone liquor following the Fehling's precipitation of a grain extract was examined chromatographically after acid hydrolysis. In this way the sugars, arabinose, glucose, galactose, and xylose were detected, in approximately that order of decreasing abundance. An investigation of the effect of the Fehling's precipitation on a barley (mother liquor) fraction (see Section I) showed that, while this preparation is precipitated as the copper complex under the conditions employed, the free gum is not recoverable from acid solution at 60% acetone.

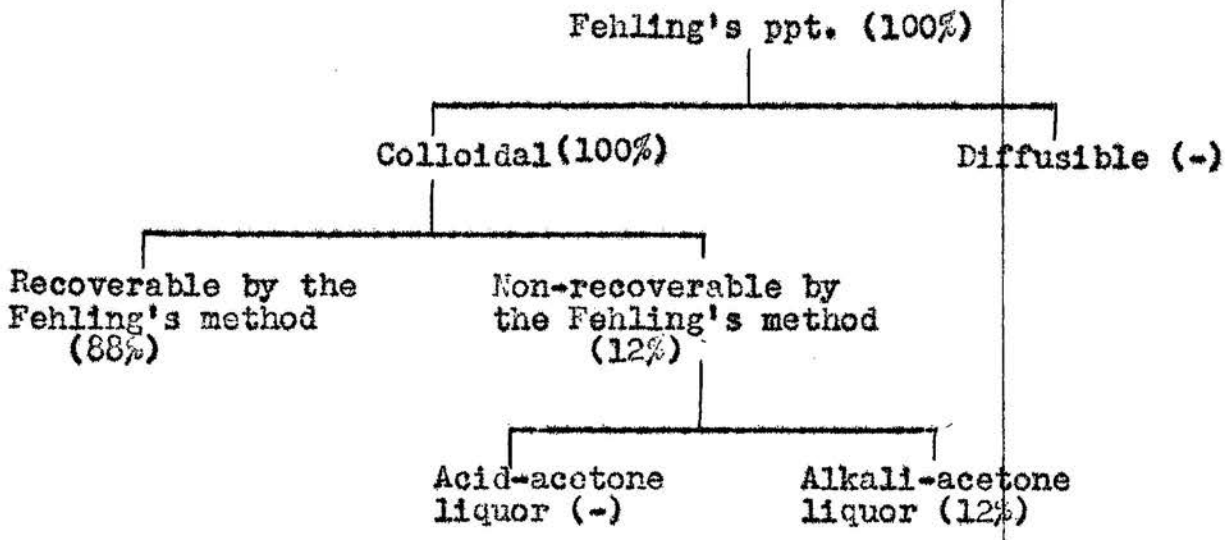


Figure XII. Result of one reprecipitation of a Fehling's precipitate.
(By the Fehling's method)

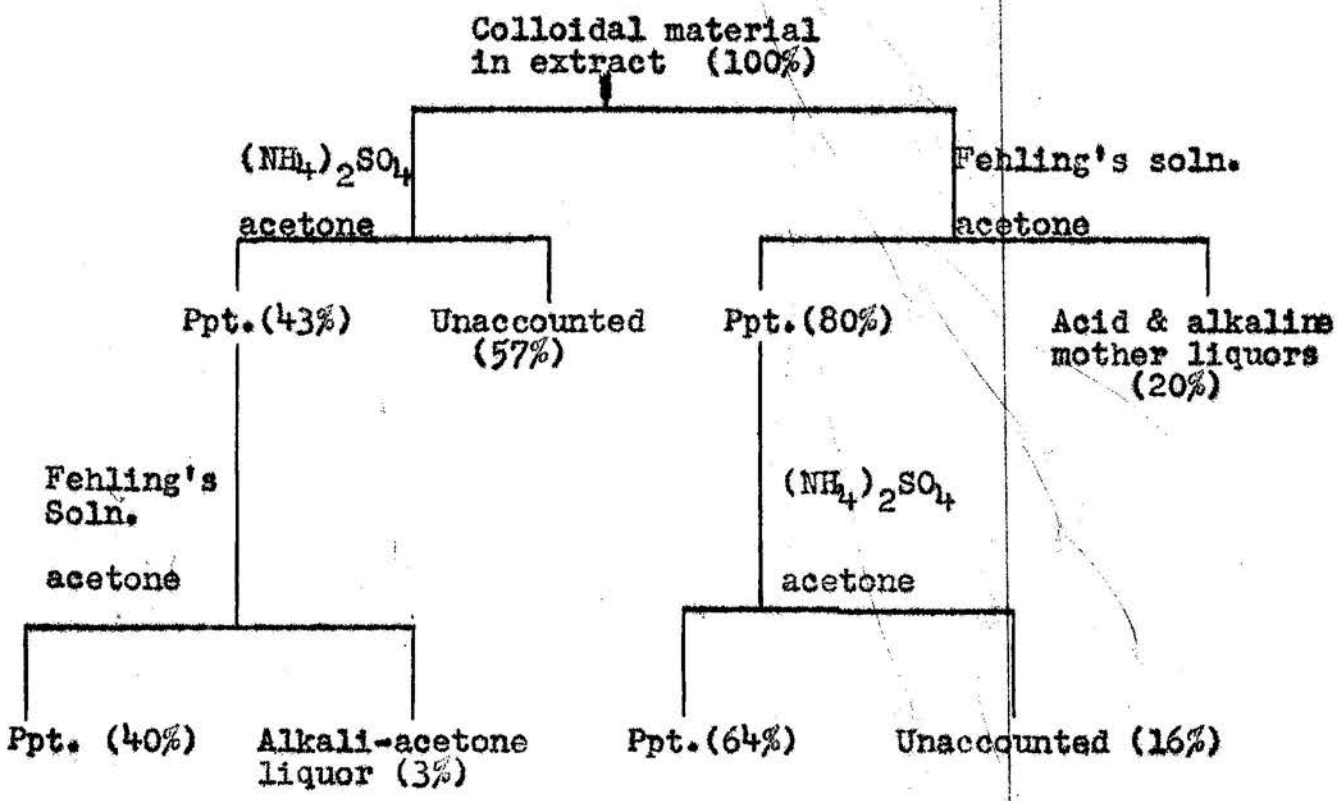


Figure XIII. Relative efficiencies of salt and Fehling's precipitations.

A comparison of relative efficiencies of the Fehling's and salt precipitations has been made. The solution of a gum prepared by the Fehling's procedure was reprecipitated with ammonium sulphate, while another gum, prepared by the salt technique, was treated with the Fehling's method. Solutions employed were 0.5% with respect to gum. In the case of the Fehling's precipitation it was found that the alkali-acetone liquor possessed substantial reducing power whereas the acid-acetone liquor exhibited none. Results can be seen in Table XXIII and Fig. XIII.

The repeated precipitation of a barley gum and a wood hemicellulose by the Fehling's method:- A sample of barley gum, in aqueous solution, was subjected to repeated Fehling's precipitation, the solution concentration in each instance being kept between the limits 0.67 and 0.69% to enable direct comparison to be made. In all, six gum precipitates were obtained, the yields at each stage estimated as glucose equivalents, and the absolute amounts of sugar units present at each stage determined by means of quantitative paper partition chromatography (butanol:acetic acid:water) in conjunction with the

Somogyi method (59). The effect of reprecipitation was also followed by means of viscosity measurement (0.5% aqueous gums solns.) at 25°C. employing an Ostwald viscometer. In order to investigate the effect of length of standing with alkali, two barley gum solutions (1A, (0.68% concn.) were allowed to stand for one, and seven days respectively, with equal volumes of Fehling's solution in stoppered flasks. The recovered gums, 2A and 3A respectively, were analysed as above. A further gum solution (1B,0.56% concn.) was allowed to stand for seven days as above, exposed to the atmosphere, the recovered gum, 2B, (one part of which had separated out from the alkaline solution) being investigated for yield and viscosity. Results are shown in Table XXIV and Fig. XIV.

A somewhat similar investigation of wood hemicelluloses H_1 and H_2 was undertaken, 0.5% solutions in 4% NaOH being used. After reprecipitation, a fraction H_1^B, soluble in acid solution, was recovered from H_1, while two fractions, H_1^A and H_2^A, were recoverable with acetone from the alkaline mother liquors of H_1 and H_2 respectively. Viscosity measurements and specific rotations were determined

TABLE XXIV

The effect of Fehling's Precipitation on a Polysaccharide Preparation and its component Sugar Units

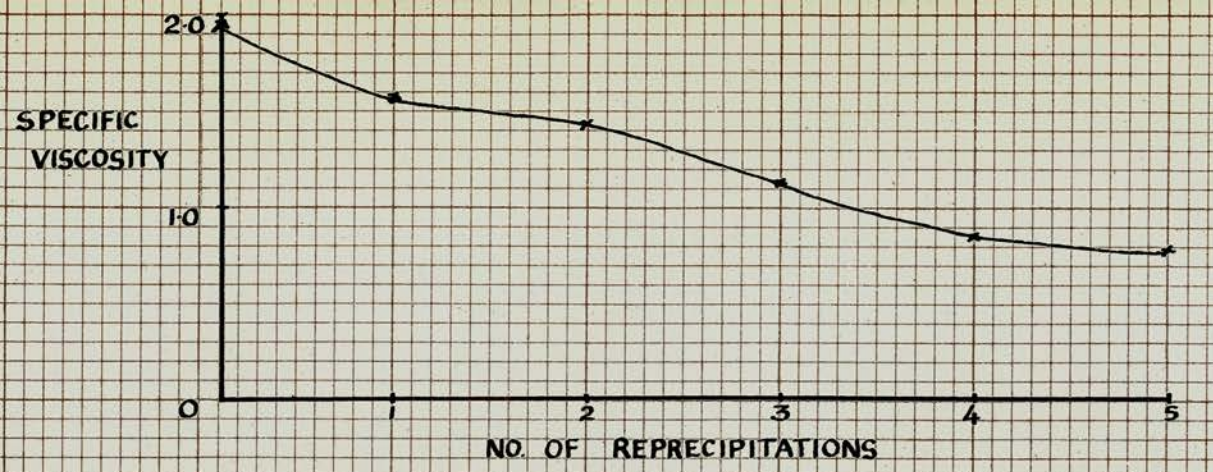
Fehling's ppt.	Recovery (% at each stage	Recovery (% of original)	Wt. of dry polysaccharide (g.)	Specific Viscosity (25°C)	Glucosan (g.)	Araban (g.)	Xylan (g.)	Total (g.) ⁄	% Recovery of sugar units*
1	91.8	91.8	1.288	1.96	0.903	0.148	0.198	1.259	97.7
2	85.6	78.6	1.183	1.58	0.911	0.070	0.176	1.157	97.8
3	86.3	67.8	1.013	1.42	0.744	0.064	0.186	0.994	98.2
4	92.2	62.6	0.874	1.13	0.725	0.044	0.085	0.854	97.7
5	90.0	56.3	0.806	0.85	0.698	0.000	0.043	0.741	91.9
6	-	-	0.725	0.79	0.558	0.000	0.073	0.631	87.0
1A	-	-	0.766	1.72	0.580	0.079	0.088	0.747	97.5
2A	92.7	92.7	0.710	1.38	-	-	-	-	-
3A	91.4	91.4	0.700	1.15	0.488	0.061	0.079	0.628	89.7
1B	-	-	0.680	1.15	-	-	-	-	-
χ2B i	8.0	8.0	0.053	-	-	-	-	-	-
ii	82.0	82.0	0.557	0.99	-	-	-	-	-

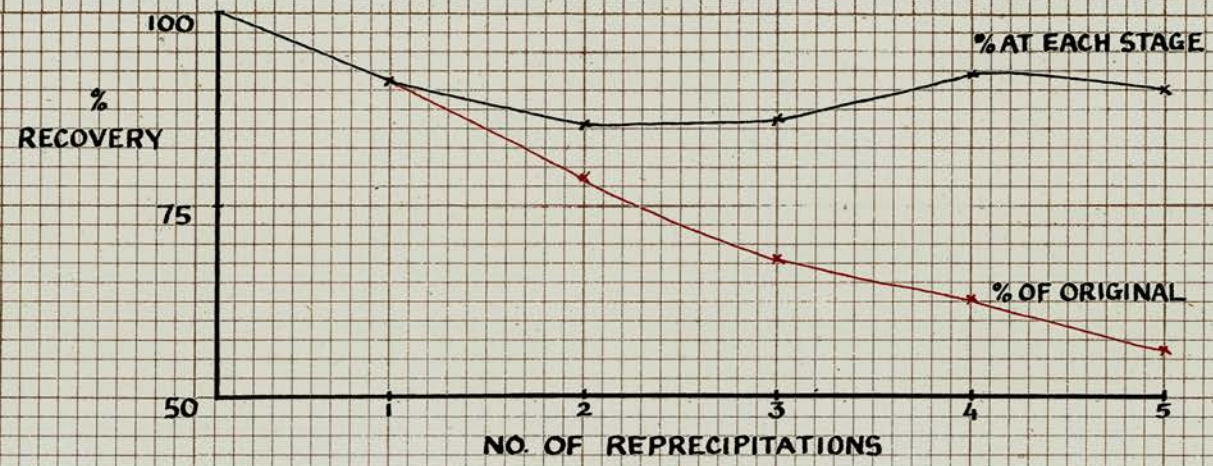
* From each polysaccharide preparation.

⁄ Irregularities due to small amounts of sugar.

χi Insoluble in alkali; (ii) pptd. from alkali with acetone; total recovery = 90%

Ppts. 1-6 represent complete solution, and recovery of ppts. obtained from 1.288g. original gum.





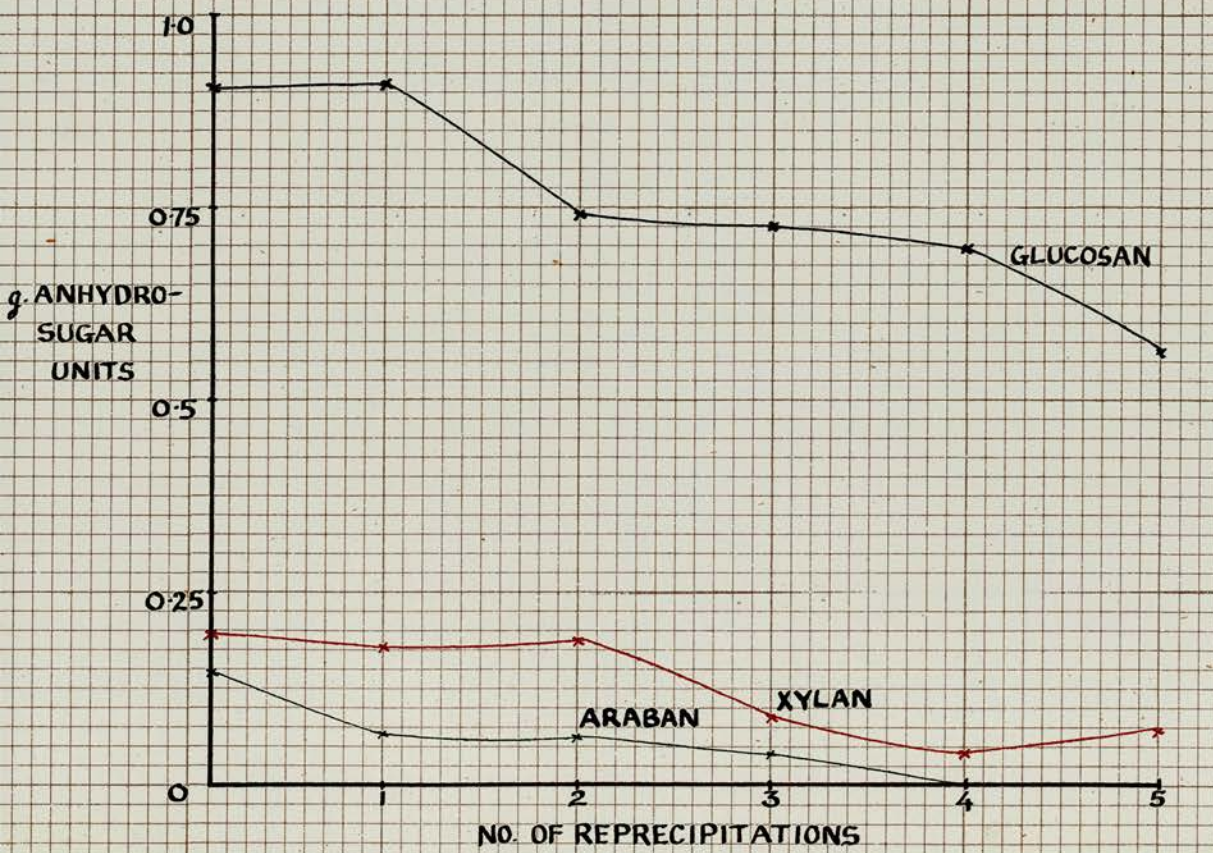


Figure XIV. Effects of repeated Fehling's precipitation on barley gum.

TABLE XXV

Analyses of Hemicelluloses at each Stage of a Fehling's Precipitation

No. of reprecipitation	Fraction	% Recovery	Specific viscosity 25°C.	Specific rotation 15°C. (°)
	H_1	-	0.11	-64
	H_2	-	0.23	-62
1	H_1	38	0.16	-
	H_1^A	1	-	-
	H_1^B	23	0.14	-58
	H_2	67	0.24	-64
	H_2^A	1	-	-
2	H_1	53	-	-
	H_1^A	ca.1	-	-
	H_1^B	29	-	-
	H_2	86	0.26	-67
	H_2^A	ca.1	-	-

TABLE XXVI

Recovery of Polysaccharides after Copper Treatment

Polysaccharide	Method of recovery	% Recovery	Specific viscosity (25°C)
Barley gum *	-	-	2.96
" "	Fehling's	90	2.27
" "	$CuSO_4$-glycerol	89	2.36
Wood hemicellulose	Fehling's	14	-
" " †	$CuSO_4$-glycerol	21	-
" " ∠	" "	1	-

* Gum before precipitation.

† Recovered as the copper complex without acetone.

∠ Recovered from the mother liquor by acetone.

on 0.5% solutions in 4% NaOH at 25°C. and 15°C respectively. Results are set out in Table XXV. These hemicelluloses yielded on hydrolysis ca.90% xylose and ca.10% uronic acid together with a trace of hexose.

Relative efficiencies of copper precipitation methods: Barley gum (0.56% aqueous soln.) and wood hemicellulose (0.5% in 4% NaOH) were treated by the Fehling's precipitation and copper sulphate-glycerol procedures as described above. In each case the gum yielded the copper complex only after the addition of acetone whereas the hemicellulose gave these without acetone. In the copper sulphate-glycerol technique the hemicellulose also yielded a very small precipitate from the mother liquor on the addition of acetone. Yields, together with viscosity measurements for the gums, are given in Table XXVI.

DISCUSSION

In a discussion of work of this nature it is much easier to state observations made than it is to put forward any definite view as to the mechanisms whereby such losses and changes are possible. Nevertheless, certain interesting points have emerged which, it is hoped, will serve to illustrate the difficulties

inherent in polysaccharide preparation, without some degree of degradation.

Copper treatment of gums:- Treatment of a grain extract with the Fehling's technique results in a distribution of initially colloidal carbohydrate between the recovered gum, the alkaline mother liquor, and the acid mother liquor. The presence of carbohydrate in this latter solution, after precipitation of a barley extract, together with its absence from this source following reprecipitation of a Fehling's precipitated gum, is of some interest. Such is suggestive of the presence in the barley extract of some polysaccharide exhibiting a certain degree of difference from that precipitable by the Fehling's method. This idea is not inconsistent with the finding, made above, that a barley mother liquor fraction* is not recoverable from acid solution by 60% acetone. Also, the sugar constituents of the carbohydrate found in the acid mother liquor, and the amount of these

*Refers to mother liquor fractions as defined in Section I and should not be confused with Fehling's precipitation mother liquors.

present, agree with values shown in Section I for the polysaccharide fractions.* These facts, together with the non-appearance of galactan in gums prepared by the Fehling's method, strongly suggest a relationship between such mother liquor gum fractions* and polysaccharide not recoverable by the normal Fehling's procedure.

Carbohydrate present in the alkaline mother liquor after a Fehling's precipitation is apparently of a diffusible nature. Furthermore, even after dialysis of a grain extract, subsequent Fehling's treatment still results in a loss of colloidal carbohydrate of approximately 10%, and this in a diffusible form. The ultimate conclusion would appear to be that the application of such a preparative procedure results in the degradation of colloidal carbohydrate. Further support for such an idea has been produced by continued application of the Fehling's technique to a gum sample. The continued viscosity decrease (although these results

*Refers to mother liquor fractions as defined in Section I and should not be confused with Fehling's precipitation mother liquors.

must be treated cautiously), coupled with gum loss at each stage, together with the virtual elimination of arabinose residues, are in agreement with the above concept. From the results of Table XXIII and Fig. XIV it is evident that glucosan material is more resistant to alkaline degradation than is the pentosan, a state of affairs also shown to hold for acid conditions (Section I). The apparent preferential removal of arabinose residues is accompanied by a decided decrease in solubility of the residual gum, solutions (both aqueous and acid) at the fourth and fifth precipitations being quite milky in appearance. This immediately recalls the effect of acid hydrolysis on such pentosan carbohydrate as described by Bywater et al. (17).

Whilst the degradative mechanism remains obscure it is not unlikely that alkaline oxidation will play some part. Length of standing of a gum in contact with a Fehling's solution, either with or without atmospheric contact, has no greater effect on gum yield or viscosity than has a straightforward Fehling's precipitation. Thus, after seven days standing in contact with the alkaline solution, whether in atmospheric contact or

not, the loss is only some 10%. whereas after one day, or on immediate precipitation, the loss is in the range of 7-15%. However, after five precipitations, involving alternate alkaline and acidic treatments, only some 56% of the original gum is recoverable in a colloidal form. Such information is suggestive of a susceptibility to alkaline conditions, either produced, or increased, by prior acidic treatment. This is all the more likely when it is considered that breakdown products occur only in the alkaline mother liquor. Such a view is remarkably close to that expressed by Sharples (81) to explain the presence of certain bonds in cellulose.

The substitution of the copper sulphate-glycerol precipitation technique for the Fehling's method, as a precipitant for the soluble gums, has not met with the success reported by Angell & Norris (3) for hemicellulose recovery. Admittedly only one precipitation has been performed in this work but it has given a yield almost identical with that obtained by a parallel Fehling's precipitation. This raises the question of the effect of inorganic ions on polysaccharides, a point which is not debatable here.

Ammonium sulphate precipitation:- It has been repeatedly remarked upon in this work that, with regard to gum yield, the salt fractionation method possesses only half the efficiency exhibited by the Fehling's solution-acetone procedure. This conclusion has now been arrived at, both by saturating a grain extract with ammonium sulphate, and by carrying out a complete fractionation. Re-precipitation of a Fehling's gum with ammonium sulphate has resulted in a recovery as high as 75% while a gum, precipitated successively six times by the Fehling's method, and presumably degraded to a certain extent, has shown an 80% recovery. This latter result serves to indicate the high molecular nature of the recoverable gum following repeated Fehling's treatment and suggests that breakdown is due to the splitting off of small fragments rather than to the large scale rupture of main polysaccharide chains. The reason for such high recoveries when Fehling's-treated gums are precipitated with ammonium sulphate is not clear although overmuch importance ought not to be attached to this since only one precipitation of each of these two types was carried out. Besides

this it has been noticed, on various occasions, that gum recoveries much higher than those reported here, after one salt precipitation, are possible.

It would appear, even at this stage in the discussion, that polysaccharide loss during salt fractionation is largely mechanical. Experience has shown that, even after six precipitations, a small portion of gum tends to remain in solution, defying recovery. Also, acetone precipitation, as carried out here, leads to losses, almost certainly of a mechanical nature. As already indicated in Section I acetone precipitation from aqueous solution results in losses depending to some extent on the nature of the polysaccharides concerned; presumably a solubility relationship, pentosan loss being greater than the less soluble β-glucosan. Despite attempts to detect polysaccharide loss from cellophane tubes during dialysis, no positive information has been gained, and it is probable that non-recovered polysaccharide has been left behind in solution at some stage, or perhaps partially retained during filtration through kieselguhr.

Comparison of the copper and salt precipitations:- Ammonium sulphate fractionation, as practised in Section I, results in a recovery of some 50% of the original polysaccharide. Although this has been stated to compare unfavourably with the Fehling's precipitation several points arise which require clarification.

The recoveries quoted for salt precipitation refer to fractionation with all its attendant re-solutions, reprecipitations, filtrations, etc. Under such circumstances direct comparison with one Fehling's precipitation is scarcely justifiable. When compared with six copper treatments it can be seen (Fig. XIV) that there is little to choose between the two techniques as regards yield. The somewhat variable gum loss on salt precipitation, regardless of the number of precipitations, is suggestive of incomplete recovery from solution under the prevailing conditions, rather than of any outright degradation.

Tables XIX and XXIV, and Fig. XIV, are suggestive of a greater sensitivity of ~~glucosan~~ pentosan material than of ~~pentosan~~ glucosan towards both these

methods. If it be assumed that the gum and hemicellulose pentosan consists of arabo-xylan of the basic structure described by Perlin (64) then mechanical loss might be expected to result in a relatively equal loss of arabinose and xylose units; always allowing for a rather greater loss of shorter chain material of different xylan: araban ratio from that of the longer chain, and perhaps less soluble, fractions. Such does not appear to be the case where continued copper treatment is concerned (Fig. XIV); indeed, the virtual removal of arabinose residues has left behind a xylan of sufficiently high molecular weight to be precipitable at 20% ammonium sulphate. Since no barley pentosan (water-soluble) is normally recoverable at this level such an observation is in keeping with decreased solubility due to the removal of arabinose units. Lower recoveries of reducing groups on acid hydrolysis of barley gum repeatedly treated by the Fehling's procedure (Table XXIV) is probably due to such a solubility decrease of the xylan residue.

It is not easy to arrive at an accurate assessment of xylan:araban ratio alteration during

a repeated ammonium sulphate-type fractionation because of the various steps involving so many fractions. With this in mind, results for hemicellulose fractionation (Table XIX) indicate the material lost to have average xylan:araban ratio very similar to that of the starting product, although in general, somewhat smaller: the recovered polysaccharide has a somewhat higher ratio than was commenced with. Such average values are not suggestive of any such change as takes place on Fehling's precipitation although they may point to a somewhat greater sensitivity of araban, than of xylan, to the salt technique.

While the method involving ammonium sulphate fractionation seems to offer the milder preparative conditions, certain unexplained rotational and viscosity changes are observed to accompany its application. The most that can be said at present is, that either of the two methods, if not overapplied, will provide a fairly accurate pattern of the sugar-unit types which constitute the higher-molecular plant polysaccharides. No major arguments should, however, be developed as to the physical properties of polysaccharides prepared by either

procedure, at least until such time as additional investigations have been performed.

Precipitation of wood hemicelluloses:- The effect of the Fehling's and copper sulphate-glycerol techniques on these substances is apparently profound as regards yield. There is considerable solubilisation of initially acid-insoluble material and also complete loss of a certain fraction. Much larger recoveries are obvious on a second reprecipitation and these might conceivably approach 100% if sufficiently extended. Little or no difference is discernible in viscosity or rotational measurements or in a chromatographic investigation of the hydrolysis products of the recovered polysaccharides. Even so, however, as has been previously pointed out, it cannot be assumed that no modification whatsoever has taken place.

PRELIMINARY INVESTIGATIONS ON CEREAL PENTOSANASE SYSTEMS

INTRODUCTION

Although a fair amount of work has been carried out on the enzymes responsible for cell wall polysaccharide metabolism the general literature does not contain a single satisfactory review of these so-called cytase enzymes. Perhaps the greatest interest thus far has been centred on the bacterial and fungal enzymes. Among these investigations may be mentioned those of Sórensen (83,84) who studied the action of a bacterial source enzyme preparation on wheat straw xylan.

With the increasing interest in the cereal polysaccharides the necessity arises for an investigation into the enzymes present in such sources, especially employing as substrates, the cereal polysaccharides themselves. Preece & Ashworth (71) made a preliminary study on the action of certain barley, germinated barley, and malt enzymes on unfractionated cereal gums and hemicelluloses. The Winnipeg Grain Research Laboratory has also published observations on barley gum-enzyme relationships (36), while from the same group of workers(8)

has come information as to the action of bacterial enzymes on unfractionated gums. Sandegren et al. (78,32), using an artificial substrate, have employed barley and green malt enzyme preparations although the dangers of such a procedure has been mentioned in the General Introduction.

With the advent of polysaccharide fractionation procedures the enzymatic hydrolysis of β-glucosan has been the subject of research by Preece et al. (74,75). There can be no doubt that the use of such a pure and natural substrate is highly desirable and has a great advantage over the earlier work. While the enzymes active on β-glucosan have been subjected to some investigation, those responsible for pentosan metabolism remain virtually untouched. Somewhat indirect information (74,44) indicates the relatively small pentosan breakdown as compared with β-glucosan. Also, information presented in Section IV, suggests the occurrence of a coupled pentosanase-transglycosidase system, thus accounting for various phenomena encountered. With this in mind, and employing rye pentosan as substrate, some preliminary enzyme relationships have been studied.

EXPERIMENTAL

Preparation of the substrate:-Rye arabo-xylan was prepared as described in Section I, the evaporation step being omitted. These preparations normally contained a very small amount of glucosan material. The fractions employed, unless otherwise stated, were those precipitable at 40 or 50% ammonium sulphate.

Preparation of crude enzymes:- Ground barley and wheat grains were separately extracted at 20°C. with 0.6% saline (15g.grain/100ml.soln.) for a period of 1 hr. Some preparations were also made by aqueous extraction but the enzymic activity of these was much smaller than in the first case. In the work described here the saline preparations were always employed. After filtration, the extracts were allowed to stand overnight to allow of autolysis of polysaccharides present, this being followed by dialysis against running water for two days, in order to remove salt and diffusible carbohydrate. A little thymol was always present to act as antiseptic. Precipitation of the extract was effected with ice-cold acetone (4 vols.) the precipitated material immediately centrifuged

off, and dried in vacuo over calcium chloride. After two days the dried material was powdered and stored as such in the refrigerator. In this way enzyme activity is retained over a period of several weeks.

Substrate solutions and enzyme extracts: Substrate solutions, either 0.5 or 1% aqueous, were prepared by constant mechanical stirring in Pyrex glass tubes with water at about 60°C. The resultant solutions were perfectly clear and normally of high viscosity. Crude enzyme preparations, in the form of acetone powders, were extracted with constant stirring in glass tubes at 25°C, using 0.6% saline, for a period of one hour. After filtration the solutions were made up to a specified volume with saline. These extracts contained 1-2.5mg. original preparation per.ml., the amount depending on the enzyme activity of the particular sample. In the work described below, unless otherwise stated, 2.5mg. were used because of the low enzyme activity involved.

Measurement of enzyme activity by viscosity drop:- These determinations were performed in the manner described by Preece & Aitken (74), incubations

being made in a thermostatically-controlled water bath at 25°C.±0.01°. Substrate solution (8ml.) and buffer (1ml.) were mixed and brought to 25°C., whereupon, 2ml. of the enzyme solution also at 25°C., were added. The reaction mixture was then introduced into an Ostwald viscometer (British Standard, time of flow for water ca. 20 sec.) and the time of flow determined at regular intervals over a period of about 90 min. Blanks were run concurrently, these containing 2ml. of 0.6% saline in place of the enzyme solution. Such blanks also furnish viscosity values at zero reaction time. For the purpose of presenting results, the reciprocals of specific viscosity were plotted against time, these curves exhibiting reasonably good linearity up to, at least, 90 min. The gradients of such curves serve as a measure of enzyme activity.

Over the initial 90 min. period the reaction shows very little dependence on the type of buffer employed; slightly greater activity is exhibited by both enzyme systems in phosphate than in acetate or citrate buffers, the latter two giving precisely the same results when incubated with the same enzyme preparation. The difference exhibited

in the presence of phosphate is much too small to be considered seriously at this early stage of the work. A few curves can be seen in Fig. XV, including one determined for a barley enzyme preparation previously treated in saline solution for 10 min. at 60°C. The difficulty in preparing pentosan solutions of equal viscosities (see Section I) has been encountered here, but does not appear to be of importance, since parallel enzyme incubations with substrate solutions of different initial viscosity yield precisely the same activity.

If Perlin's idea (64) be taken to hold, at least in a general sense, for these arabo-xylans, it seems likely that the enzymically-induced viscosity drop will be mainly a result of the splitting of the main xylan chain. The pH conditions under which this enzyme, or enzyme system, functions, were investigated as above, at various pH values, in sodium citrate-HCl, and sodium citrate-NaOH, buffer solutions. A pH/activity curve, constructed for the barley enzyme system and a 1% substrate solution, is shown in Fig. XV. A few similar measurements, performed at 0.5 and 2.0% substrate concentrations, indicated no change in the optimum

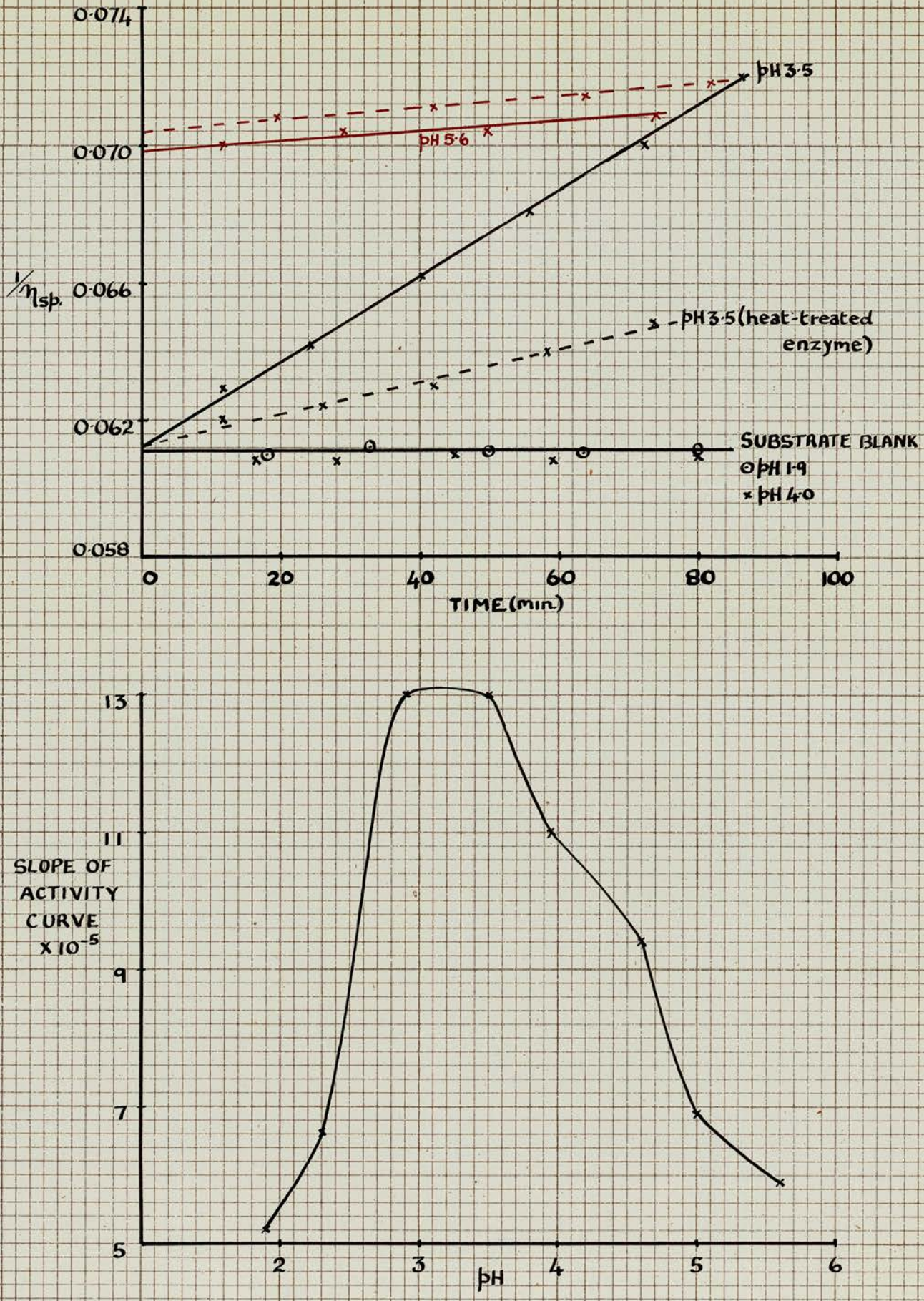


Figure XV. Activity of cereal pentosanase ('xylanase') systems.
Black curves-barley system (citrate buffer)

Red curves-wheat system (full line, citrate; broken line, phosphate)

pH which can be seen to lie between pH values of about 2.9 and 3.5. In view of the somewhat labile nature of this pentosan substrate it was necessary to carry out careful control experiments, more especially at the lower pH values. It was found, that, over the experimental pH range, the substrate was completely stable with respect to viscosity measurement (Fig. XV.).

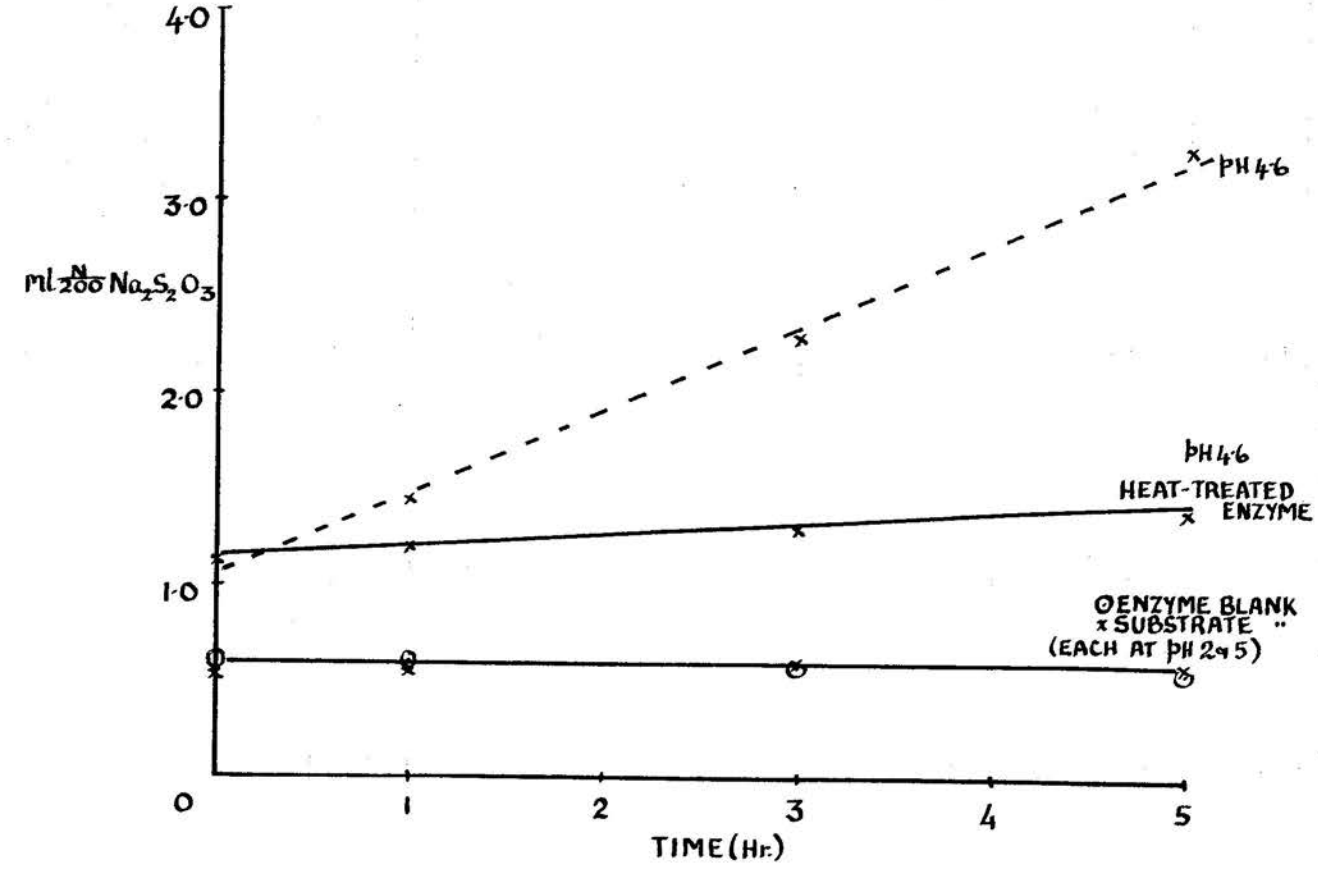
Measurement of enzyme activity by determination of reducing group liberation:- Incubations were made at 25°C. in Pyrex glass tubes using 2ml. of 1% substrate solution, 1ml. of the enzyme solution (containing 2.5mg.original crude material), and 0.25ml. of buffer. Enzyme blanks contained 2ml. water in place of the substrate solution, and gum blanks had 1ml. saline in place of the enzyme solution. Water blanks, containing 3.25ml., were run simultaneously. On completion of incubation, 3ml. of Somogyi reagent (59) were measured into each tube, and reducing power determined in each case. Allowance was made for reducing groups due to the enzyme preparation, and the original polysaccharide, thus providing values for the enzymic liberation of reducing groups; reducing power at zero time was

determined on suitable portions of the substrate and enzyme blanks. Reactions were followed over a five hour period, and curves were constructed, a few of which are shown in Fig. XVI.

In order to investigate the pH/activity relationship for reducing group liberation, incubations were carried out as above, over a pH range employing citrate buffer. Two separate experiments, one of incubation time 3hr., and the other of 5hr., were run; the 3hr. run employed substrate which was virtually uncontaminated by glucosan. pH/activity curves for these are presented in Fig. XVI.

The effect of enzyme action on ammonium sulphate precipitability of rye arabo-xylan:- 100ml. portions of 0.5% gum solution were incubated for 48 hr. at 25°C. under the conditions given in Table XXVII. It should be noted that the weights of barley and wheat enzyme preparations employed are in the relative proportions obtained from the respective grains.

Enzyme action was terminated by bringing each mixture to boiling point and maintaining thus for 1-2 min. After cooling to room temperature, each solution was fractionated with ammonium sulphate



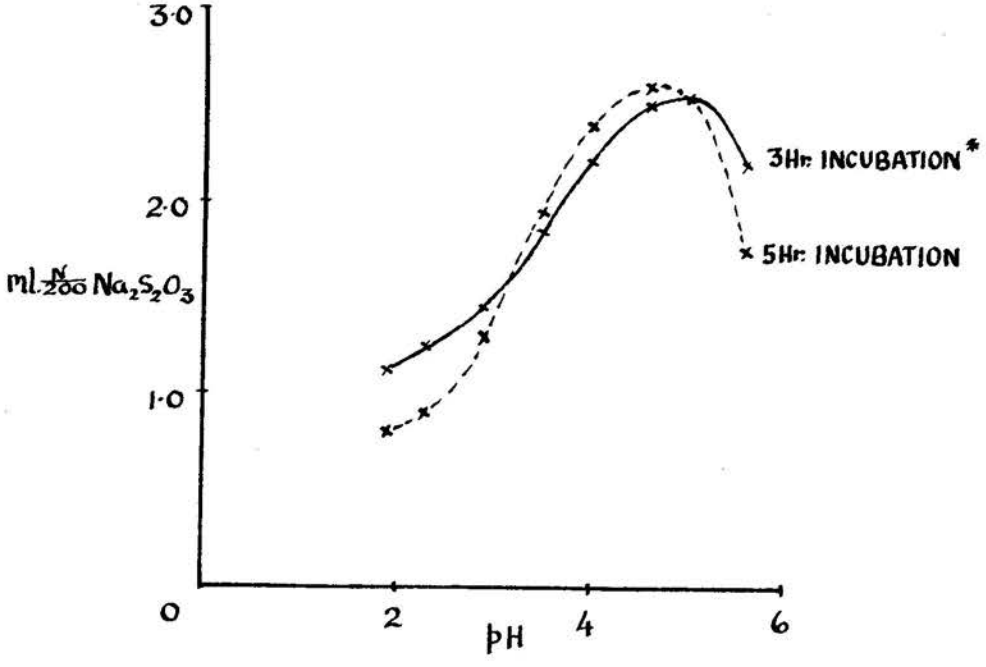


Figure XVI. Liberation of reducing groups by the barley pentosanase system(citrate buffer).

* Glucosan-free substrate.

TABLE XXVII

Incubation Systems

Flask No.	Gum soln. (0.5%) (ml.)	Enzyme soln. (ml.)	Acetate buffer pH 4.6 (ml.)	0.6% saline (ml.)	KH_2PO_4 molarity (final)
1	100	20*	10	-	0.01
2	100	20*	10	-	-
3	100	20x	10	-	0.01
4	100	-	10	20	-

* Wheat enzyme (7mg./ml. original preparation)

x Barley enzyme (4.5mg./ml. " ")

TABLE XXVIII

Gum Recoveries after Enzyme Action

Flask No. *	Precipitation level (% salt)	Recovery (g.gum)	Recovery (% of original)
1	30 40	0.219 0.127	74.5
2	30 40	0.090 0.105	42.2
3	40 50	0.168 0.040	45.0
4	30 40	0.310 0.028	73.2

* See Table XXVII.

in the normal manner, except that, in view of the small amounts of polysaccharide involved, it was advisable to carry out only one precipitation in place of the normal four or six; this was followed by dialysis and recovery as usual. Small precipitates obtained from each of the flasks 1, 2, and 3, at 20% salt concentration, probably consisted of protein material; at the 30% level, flasks 1, 2, and 4, yielded precipitates, but none was obtained from flask 3; at 40%, precipitation was produced in flasks 1, 2, and 3, with a very small recovery from 4; at 50% only flask 3 yielded precipitation (small) this differing from those obtained hitherto by reason of its pulverulent nature, the others being fibrous. Gum recoveries are given in Table XXVIII.

For the purpose of investigating any change undergone by the xylan:araban ratio of the pentosan during enzyme action, acid hydrolysates of the recovered gums were partitioned in butanol-ethanol-water (45-5-50, 1% by wt. of NH_3 in aqueous phase; Whatman No. 1 paper). Arabinose and xylose were separately determined after elution (Section I) and their ratios calculated, as shown in Table XXIX. Figures are also given for 'xylan' and 'araban' recoveries in each case.

TABLE XXIX

Effect of Enzyme Action on the Xylan:Araban Ratio of Rye Arabo-Xylan

Flask No. *	Precipitation level (% salt)	Xylan:araban ratio	Xylan Recovery (g.)	Araban Recovery (g.)
1	30	2.23	0.151	0.068
	40	1.56	0.077	0.050
2	30	1.78	0.058	0.032
	40	1.78	0.067	0.038
3	40	1.63	0.104	0.064
	50	1.70	0.025	0.015
4	30	1.78	0.198	0.112
	40	1.44	0.017	0.010

* See Table XXVII.

Chromatographic investigation of enzyme action:-
In the first instance, substrate and enzyme, with or without buffer, were incubated together at 25°C. for varying times of 1, 3, 5, and 48 hr. On completion of incubation, the non-decomposed pentosan was precipitated with acetone, filtered off, and the filtrate taken to dryness in vacuo (40°C). The dry residue was taken up in two drops of water and chromatographed on paper in butanol-acetic acid-water, or butanol-ethanol-water. This procedure was not entirely satisfactory due to the relatively high concentration of carbohydrate necessary to show the presence of the lower-molecular material, a state of affairs which resulted in the building up of such a concentration on the starting line, as to cause a marked retardation of the products. Even so, however, it was perfectly obvious from several chromatograms that the first visible product was arabinose. This gradually increased in amount, with oligosaccharides and xylose making their appearance. Over the initial reaction period arabinose remained the predominant product.

It became fairly obvious that long incubation times were necessary in order to detect the

reaction products of these enzymes of low activity, the following procedure being adopted. Substrate (1% soln.) and enzyme (2.5mg./ml., original material) were incubated together, without buffer, in the presence of toluene and thymol for 48hr. periods at 25°C. The proportions were those employed in the experiments on reducing group liberation, namely 2:1; 6ml. substrate, and 3ml. enzyme solution were normally taken. Enzymes employed were those from barley and wheat, together with that from barley, previously heated in saline solution at 60°C. for 10 min. Enzyme controls contained water in place of substrate solution, and a substrate blank, containing saline in place of enzyme, were run concurrently. Reactions were terminated by heating in a boiling water bath for 2 min., non-decomposed gum being precipitated, on cooling, with two volumes of ethanol. In an attempt to obtain clearer chromatograms, the general procedure described by Sörensen (83) was employed. The alcoholic filtrate was taken to dryness in vacuo (40°C), the residue taken up in 2 ml. of 30% ethanol, and this solution centrifuged to remove any residual higher-molecular material. The centrifugate was then

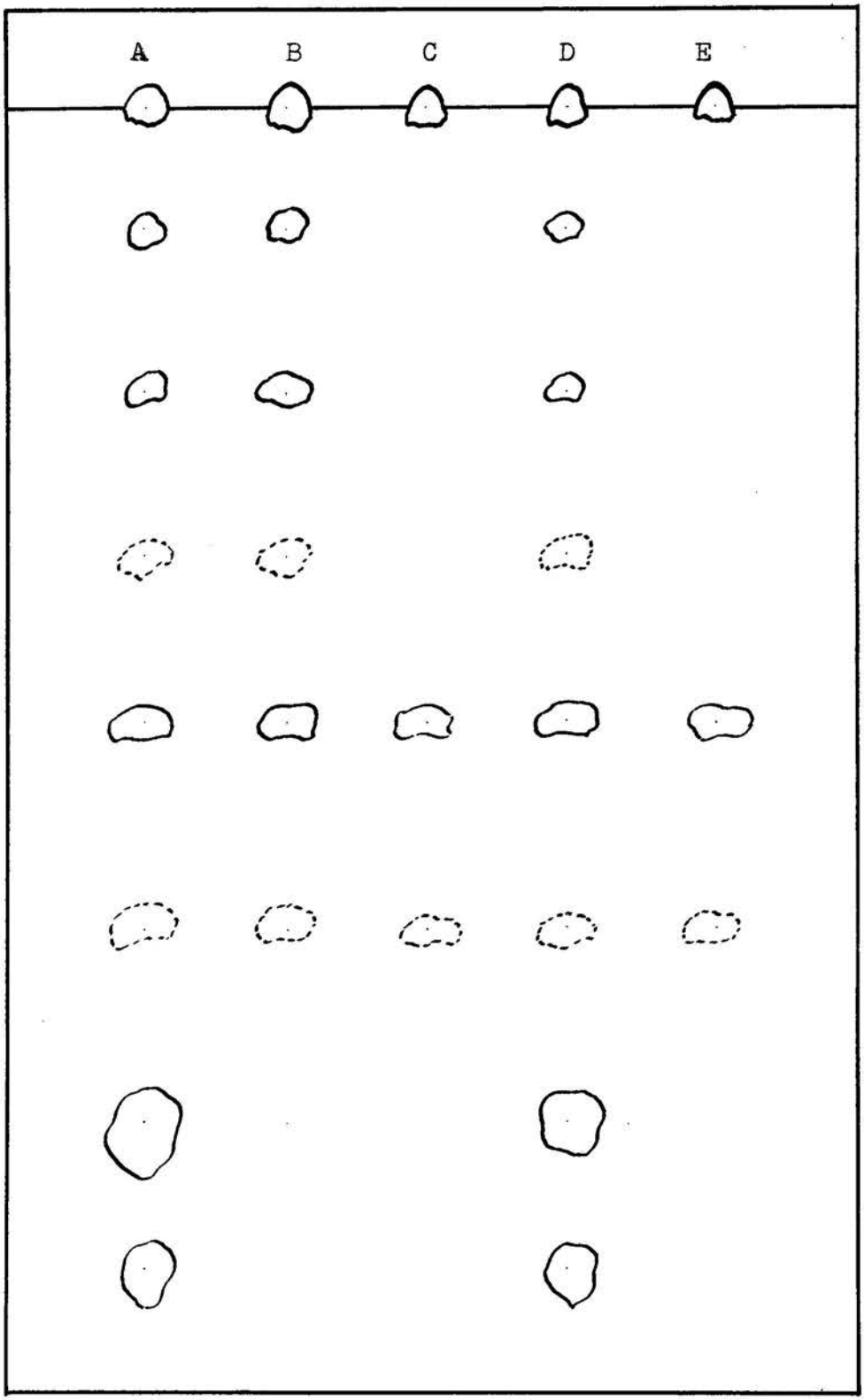


Figure XVII. Chromatogram of pentosanase action.

A barley system

B ditto, with 60°C treatment

C barley system(blank)

D wheat system

E wheat system(blank)

Substrate blanks showed no mobile carbohydrates.

TABLE XXX

Products of Cereal Pentosanase Action

Reaction mixtures			Enzyme blanks		Gum blank	Rf value
Barley enzyme	Barley enzyme (60°C.)	Wheat enzyme	Barley enzyme	Wheat enzyme		
+++	++	++	+	+	-	0
+	±	-	-	±	-	0.02
+	+	-	-	±	-	0.05-0.06
++*	±*	++*	-	-	-	0.09
++	++	+	+	+	-	0.12
+*	±*	+*	+*	+*	-	0.17
++	-	+	-	-	-	0.21
+	-	+	-	-	-	0.24

+++ Major component

++ Minor "

+ Trace.

± Uncertain presence.

- Apparently absent.

* Hexose indicated.

taken to a small volume in vacuo (40°C). Partition chromatography was carried out on paper in butanol-acetic acid-water against known sugars. While the spots obtained with aniline oxalate reagent were not sufficiently distinct to allow of successful photography, much superior separation was obtained than in any of the previous cases. A diagram (to scale) of such a chromatogram is presented in Fig. XVII., and a description can be seen in Table XXX.

DISCUSSION

General:- Whilst acknowledging that all enzymic studies possess a certain degree of difficulty, it is considered that the cereal pentosanase problem is somewhat more acute than is that of the β-glucosanase system. That this is so, is due, first of all, to the difficulties involved in preparing a glucosan-free substrate in comparison with the much more readily available β-glucosan. Quite apart from this problem, however, the activities of the two enzyme systems are obviously very different, the very low pentosanase activity making necessary much longer incubation times for the identification of reaction products.

Notwithstanding the above-mentioned

difficulties, a few interesting points have emerged from the present work. Observations made will not only serve as a basis for future work; they have already been fairly successful in supporting various points which have previously arisen. In what is said below, sight is not lost of the fact that this investigation was conducted with crude enzyme preparations which were probably of a somewhat lowered activity due to precipitation procedures and storage.

The barley pentosanase system:- As has already been said, it is likely that enzymically-induced viscosity drop in the pentosan substrate is due to the splitting of main xylan chains. There seems every likelihood that such viscosity decrease is a manifestation of the activity of a component present in the barley system, which component may conveniently be termed a 'xylanase', and which may be rather similar to that described by Sórensen (83) to be present in a bacterial source. This barley 'xylanase' is obviously of great interest, exhibiting as it does, optimum activity between pH 2.9 and pH 3.5, a very low value for a plant enzyme. Moreover, the activity at pH 2.3 is greater than that at pH 5.6, a remarkable feature.

Preece and Ashworth (71) indicated the presence, in cereal grains, of two enzyme types, namely, a cytoclastic or disaggregating enzyme, and a cytolytic or saccharifying enzyme. Attempts to investigate optimum conditions for reducing group liberation in the present work have been attended by doubtful success due to the presence of a small amount of dextrinous material in the substrate. The β-amylase of the enzyme preparations is active on this and, over the initial reaction range at least, its activity is greater than that of the pentosanase system. Such a state of affairs has undoubtedly resulted largely in the measurement of β-amylase activity with a pH optimum of approximately 4.5. Nevertheless, one incubation, carried out with a pentosan substrate virtually uncontaminated by glucosan, shows a pH/activity curve of slightly higher optimum pH (Fig. XVI). Whether this is evidence for the occurrence of a further enzyme in addition to the 'xylanase' is uncertain.

Useful if somewhat scanty information on this latter problem has been obtained by chromatographic studies. The action of untreated barley and wheat enzymes suggests the initial reducing group

liberation to be due to the release of arabinose. Oligosaccharides which make their appearance later in the reaction include xylobiose as the chief product. Although the others were not subjected to individual analysis, the presence of both xylose and arabinose was ascertained by acid hydrolysis of the total. This suggests the presence of mixed oligosaccharides similar to those found by Bishop et al.(10) after the enzymic degradation of a wheat straw xylan. These oligosaccharides persist after treatment of the enzymes at 60°C. for 10 min., conditions which apparently inactivate the factor (or factors) responsible for the liberation of arabinose and xylose. Such information constitutes much stronger evidence for the existence of at least one other enzyme or enzyme system, besides 'xylanase'. The general picture built up here is not at all dissimilar to that presented by Sórensen (83) for his bacterial enzymes.

The relatively early appearance of arabinose among the reaction products may well mean that its removal facilitates 'xylanase' action on a xylan chain possessing fewer branches than before;

in effect, a more 'natural substrate'. Lowered activity of heat-treated enzymes, as regards viscosity drop, could perhaps be explained in such a way, although the 'xylanase' itself may not be entirely unaffected by the heat treatment.

While it would be a mistake to over-hypothesise with the results available, a few points seem worthy of consideration. A barley 'xylanase', which exhibits some resistance to heat, is active in splitting the main xylan chain of the arabo-xylan molecule, particularly after the removal of arabinose by a second enzyme system, to give oligo-saccharides of varying chain length. This is apparently followed by the action of an 'oligo-saccharidase' liberating xylose, and possibly arabinose, from the oligosaccharides, this system being heat-sensitive. The system responsible for the initial arabinose removal, if it differs from the 'oligosaccharidase', is similarly affected by heat. It should not be supposed that the possibility of xylose removal direct from the polysaccharide chains has been ruled out.

The wheat pentosanase system: Little can be said except that the general reaction pattern seems very

similar to that of the barley system; however, barley 'xylanase' activity is some seven times greater than that of the corresponding wheat system. Preliminary work (76) seems to indicate very little difference between the two systems in respect of reducing group liberation, though this must be considered very carefully in the light of what has already been said about the β-amylase activity. Nevertheless, parallel chromatographic studies do suggest similar initial liberation of arabinose in both cases.

The significance of the present work in the light of previous knowledge:- That free pentoses make only a fleeting appearance during the malting of barley (44) may mean that, although they are produced, they are employed in the resynthesis of new, higher-molecular carbohydrate. It has already been suggested (Section IV) that an enzyme system concerned with the transport of arabinose between pentosan chains, and thus controlling solubility relationships, might be active, particularly in the wheat grain (77). Such a system could conceivably be responsible for the resynthesis mentioned; at least there can be no doubt as to the availability of free arabinose as soon as enzyme action occurs.

The data presented in Table XXX is good supporting evidence for the large barley 'xylanase' activity compared with that of wheat. Pentosan originally precipitable at 30% ammonium sulphate can no longer be recovered at this level after barley enzyme action although at least half the polysaccharide recoverable after wheat enzyme action is still precipitable at this concentration. An apparent decrease in chain length (as judged by water-solubility) produced by the barley system is accompanied by a change in the xylan:araban ratios in such a way as to form a trend already shown to hold for salt-precipitated barley gums (72), i.e. an increasing ratio with increasing solubility; although it must be realised that overmuch importance should not be attached to this since only two fractions are available. The wheat enzyme system exhibits interesting behaviour in the presence of added inorganic phosphate, as much gum being recoverable as in the control experiment. There is, nevertheless, evidence for some increased solubilisation which has apparently come about through arabinose transfer since the gum recoverable at 30% salt concentration has a substantially increased xylan:

araban ratio. Indeed we have here the beginning of a trend seen in the wheat gums described by Perlin (64) and in those of Section I in this work, namely, a xylan:araban ratio decreasing with increasing solubility. In the absence of added phosphate no such trend is visible, the ratio remaining constant in the two fractions recovered, although in this case recovery of high-molecular polysaccharide is much less. On the basis of these results the arabinose transfer reaction would appear to be phosphate-dependent. These findings could alternatively indicate phosphate inhibition of the 'xylanase' although this seems less likely owing to the slightly greater viscosity drop in the presence of added phosphate. This latter observation seems to be in accordance with the idea of increased arabinose removal, and hence, greater 'xylanase' activity, in the presence of added phosphate. It is not known what effect phosphate has on the barley system since no experiment of long incubation time was performed in the absence of the phosphate. Interest resides in the observation that, where phosphate has been added, the higher-molecular reaction products of both systems form the beginning

of a trend shown by the wheat and barley gums; this is not so for the wheat system in the absence of added phosphate. The possible importance of phosphate must not be overlooked in subsequent work of this nature. The possibility of variable gum recovery owing to ammonium sulphate precipitation has not been overlooked in this investigation. While this danger does exist, all precipitations of this type were carefully standardised, and are believed to be directly comparable.

While much that has been said above is of a speculative nature this work must form the basis for future investigations of the enzyme problem. There is here a fruitful field for research, and one moreover, that should adequately repay the work expended upon it.

GENERAL DISCUSSION

The water-soluble polysaccharides:- The preparations described in Section I were undertaken using a fairly standard technique and therefore do not lack a basis for direct comparison of the products. It is felt, however, that such a comparison can only be justifiably applied to the sugar unit types concerned, it having been made abundantly clear that strict physical measurements may lead to erroneous conclusions. With this proviso in mind it seems fairly certain that the true non-starchy water-soluble cereal polysaccharides consist of a somewhat pulverulent arabo-xylan of high negative rotation, and a more fibrous β-glucosan of low negative rotation. The relative amounts of these present depend on the cereal grain concerned, and within any one grain, on varietal considerations etc. Besides the above-mentioned polysaccharide types, there exist small amounts of glactan in one form or another, and also perhaps, an araban; these, however, have scant claim to be called cereal gums. Also present in the gum preparations, again depending to some extent on source, are variable amounts of α-glucosan, evidently derived from starch. These three polysaccharide types, α- and

β-glucosans, and arabo-xylan, seem to explain the methylated carbohydrates obtained by Gilles et al. (34) from a barley gum preparation.

It has been pointed out (49) that simple alcohol treatment, as carried out above prior to gum extraction, does not result in complete enzyme inactivation. Sight has not been lost of this danger when it is stated that the prepared polysaccharide fractions represent a fairly accurate picture of the sugar-unit types occurring in the raw grains. Experience suggests that the residual enzymic activity may cause no more alteration to the gums than will certain of the preparative techniques themselves. The large viscosity differences observed for rye pentosan preparations obtained with and without the rather drastic evaporation step described in Section I, are not inconsistent with such a view.

It is not yet certain whether arabo-xylans from different sources possess similar structures. Information obtained from acid hydrolysis, enzymic treatment, specific rotation, etc., indicates at least a similar basic structure in the cases investigated, namely, a xylan chain carrying arabinose residues as side chains. Variability in chain

length and araban:xylan ratio apparently occur to an extent governed by the type of grain.

The β-glucosans of barley and oats are very similar with respect to the determinations carried out. Both are much less susceptible to acid hydrolysis than is the arabo-xylan and the presence of cellobiose and laminaribiose in the hydrolysates of each, is evidence for the occurrence of 1, 3 and 1, 4 β-linkages in both cases. The non-precipitability of arabo-xylan at 20% salt concentration facilitates the recovery of β-glucosan in an uncontaminated form. This solubility difference exists to a certain extent in the cereal hemicelluloses.

The cereal hemicelluloses:- The hemicelluloses examined, namely, those which are more easily extracted from the grains, exhibit, on the whole, similar properties to those of the water-soluble gums. Indeed it seems unlikely that there is any fundamental difference between the two types as regards structure. An arabo-xylan, apparently of similar structure to that of the cereal gum pentosan, and a glucosan, possibly related to the water-soluble β-glucosan, emerge as constituents of these fractions. Uronic acid participation in the hemicellulose structure, while greater than that in the gums, is

still fairly small; significant amounts are found in the husk polysaccharide. Whether uronic acid groups are active in cell wall combination seems worthy of consideration. The hemicellulosic glucosan, apparently concentrated in the grain endosperm, seems to possess a similar specific rotation to the barley β-glucosan, and has also been shown to contain, at least, 1, 4 β-linkages.

Thus far, any sharp distinction between the gums and hemicelluloses, besides the difference in initial solubility, can scarcely be justified, and is perhaps more apparent than real. The solubility difference may prove to be a result of the extent of cell wall combination, whether through primary or secondary forces. One must alsc bear in mind the possibility of salt formation, as in the case of calcium pectate. The relative ease with which these hemicelluloses (Section IV) can be coaxed into aqueous solution after isolation, is suggestive of initial insolubility of the polysaccharides themselves. The hemicelluloses recovered in this present work presumably represent that fraction which is most weakly held.

Over and above the similarities existing in the

general constitution and properties of the gums and easily extractable hemicelluloses, rather similar behaviour is exhibited on salt fractionation. Thus, in both cases, β-glucosan content of the salt-precipitated fractions tends to decrease with increasing water-solubility, the pentosan behaving in a reciprocal fashion. Where anomalies arise, e.g. for the barley and wheat gums, glucosan content rising in the more soluble fractions, dextrinous contamination appears to be the cause.

The husk-type hemicellulose, a urono-arabo-xylan, which has been generally assumed to perform a predominantly structural role, conforms to the 'typical hemicellulose' suggested by Preece & Ashworth (71). Besides this, an initially insoluble gum-like hemicellulose fraction (presumably the endosperm hemicellulose of the present work), and of course, the initially soluble gum, were postulated by the same workers (71). In this respect the two investigations show some measure of agreement.

Hemicellulose-gum relationships in the cereal grains: It seems very likely that β-glucosan, in the cereals where it exists, is primarily in the nature of a reserve polysaccharide. The rapid increase of the

water-soluble β-glucosan fraction during autolysis (74,77) is believed to arise from the initially insoluble fraction of the endosperm. While this is very likely, it should be borne in mind that certain transglycosidation steps, mentioned by Preece et al (75) and Enebo et al.(32), may introduce complications into the idea of a simple solubilisation. Small amounts of glucosan occurring in the husk hemicellulose preparations probably represent a shorter chain 'cellulosan' of structural importance.

The higher-molecular cereal grain pentosan might conceivably fulfill a more structural role; consistent with 'typical hemicelluloses' of woods and straws, which are predominantly pentosans or urono-pentosans. The slight increase in water-soluble barley pentosan during autolysis (2) is in keeping with the low pentosanase activity of this cereal (Section VI) in comparison with the corresponding glucosanase system (74). The observed increase is most probably due to enzyme activity on initially insoluble pentosan located in, or near, the endosperm; that portion undergoing early solubilisation perhaps corresponding to the more weakly held hemicellulose prepared in Section IV.

Both this hemicellulose portion and the soluble gums themselves seem to be concentrated in the endosperm.

The husk polysaccharide is unlikely to contribute to the solubilising process to anything approaching the same extent as do the highly viscous endosperm materials; if at all. The possibility of some degree of equilibrium between the husk pentosan and the endosperm-type pentosan must not be dismissed, even if only because of their similar properties. However, it is felt that any such equilibrium must favour the husk polysaccharide, as shown below. Any such relationship between the initially soluble gums and the husk hemicellulose probably exists through the medium of the endosperm hemicellulose.

Urono-arabo-xylan ⇌	arabo-xylan ⇌	arabo-xylan
(husk)	(endosperm)	(endosperm)
'Typical hemicellulose'	Gum precursor hemicellulose	Water-soluble gum

It would be of major interest to determine the precise site of location of the pentosan in the endosperm. In this way it might be easier to explain any relationship between it and the husk polysaccharide.

Importance of the cereal arabo-xylan: The investigation conducted above suggests an important central role for this pentosan, perhaps not only in cereal biochemistry, but also in cell wall metabolism throughout the plant kingdom. It was originally proposed by Perlin (64), in view of his finding concerning the relationship between xylan:araban ratio and solubility, that the occurrence of arabinose residues in the form of side chains conferred upon a xylan molecule a means of easy transport within the plant; removal of arabinose units would then result in the deposition in the cell wall of structural xylan of decreased solubility. There is now ample evidence for the availability of free arabinose at the onset of cereal (at least barley and wheat) pentosanase activity, and additional information suggests a mechanism for arabinose transfer between xylan chains. It has already been pointed out in the General Introduction that there is evidence for the existence, in plant tissues, of two xylan types, one with, and one without, arabinose residues. That such a state of affairs may well

hold is not denied, but in view of what has been said regarding arabinose transfer, the possibility arises that the presence or otherwise of 'true xylan' will be a reflection of the enzyme system of the plant, or plant tissue, concerned. It seems likely that 'xylan', as such, will be found mainly in structural tissues such as woods. Such a state of affairs would represent one extreme in arabo-xylan metabolism, namely, that of highest xylan:araban ratio. Xylans of this type, will presumably be less easily mobilised through an arabinose transfer system, being as they are, more permanent cell wall substances. The endosperm 'gum precursor hemicellulose:' would appear to offer an excellent material, either for direct formation of 'structural hemicellulose', or for soluble gum production followed by translocation and subsequent reversion to husk-type, or woody, polysaccharide.

In view of this possible central role for arabo-xylan in cereal biochemistry it is well to bear in mind a word of warning as to preparative methods. The effect of alkaline treatment on the arabinose side chains of arabo-xylan must not be

overlooked, especially where conclusions regarding plant metabolism are to be drawn from the resultant preparations. A small araban content could easily be removed unnoticed under such circumstances. Chemical structure of 'xylans' prepared by repeated Fehling's precipitation are no doubt accurate, but can they always be truthfully termed 'plant cell wall polysaccharides'?

PUBLICATIONS

The material of Section I has been published in a paper entitled "Higher Molecular Gums of Common Cereals", (J. Inst. Brew., 385, 1953)

A similar publication has been made of the contents of Section IV: "Some Hemicellulose Fractions", (J. Inst. Brew., 490, 1954)

"Electrophoresis of Polysaccharides" has been communicated to Chem. & Ind., this representing the work of Section II.

It is hoped to publish the method for uronic determination (Section III), and also the enzyme relationships described in Section VI.

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